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The Intracellular Antibody Capture Technology (IACT): Towards a Consensus Sequence for Intracellular Antibodies

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We describe the application of an intracellular antibody capture technology (IACT) as a generic *in vivo* selection procedure for isolating intracellular antibodies or ICAbs. IACT was applied to the *de novo* selection of functional ICAbs against the microtubule-associated protein TAU, found in neurofibrillary lesions of Alzheimer's disease brains. A panel of 17 different ICAbs was created which bind TAU inside cells and the epitopes recognized by the selected ICAbs have been determined by an *in vivo* epitope mapping procedure. Finally, sequence analysis showed that the IACT-derived ICAbs are characterized by a common signature of conserved amino acid residues, suggesting that the IACT naturally selects a sort of ''captured consensus sequence'' for intracellular antibodies. The development of IACT, together with the possibility of scaling up in a high throughput and automated format, makes IACT a new enabling tool for target validation in functional genomics and global proteomics.

Keywords: intrabodies; single-chain Fv; functional genomics; TAU; Alzheimer's disease

Introduction

Expression of antibodies as single chain Fv fragments (scFv) inside cells has been successfully used to ablate protein function.[1] Thus, intracellular antibodies (ICAbs) should play a role in human disease therapy by interfering with abnormal protein activity. Furthermore, in the context of functional genomics projects, ICAbs promise to be an important tool to knocking-out protein function inside the cell. In particular, technologies to achieve protein knock-out at a genomic scale would provide an essential tool to complement the growing efforts at building exhaustive protein interaction maps of cellular networks.[2–5] In this context, however, the need for speed and high throughput requires the development of simple and robust methods to derive antibodies which function within cells, without the need for optimization of each individual ICAb. When antibodies are expressed in the cytoplasm, folding problems often occur.[6] The reducing conditions of the cytoplasm hinder the formation of the intra-domain disulphide bonds in the antibody variable domains.[7,8] However, some scFv fragments tolerate the absence of this bond.[9,10] No rules or predictions however can yet be made, about which antibodies will fold in the cell cytoplasm. We have recently provided the proof of principle of the intracellular antibody capture technology (IACT), an *in vivo* selection strategy for functional ICAbs, based on a two-hybrid approach.[11] IACT promises to be a robust procedure that should facilitate the isolation of specific ICAbs from complex mixtures. The technology has subsequently been used for the isolation of putative intracellular binders, but no functional characterization of the intracellular antibodies was provided.[12,13] Here, we demonstrate that IACT can

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Abbreviations used: ICAb, intracellular antibody; IACT, intracellular antibody capture technology; scFv, single chain Fv fragment; IVEM, *in vivo* epitope mapping; ICS, intrabody consensus sequence; AD, Alzheimer's disease; MAP, microtubule-associated protein; MBP, maltose-binding protein; NLS, nuclear localization signals; CDR, complementarity-determining region; VH, variable domain of the antibody heavy chain; VL, variable domain of the antibody light chain; ELISA, enzyme-linked immunosorbent assay.

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be effectively applied to the *de novo* selection of panels of functional ICAbs against diverse protein antigens and report the full characterization of a set of ICAbs selected against the microtubule-associated protein (MAP) TAU, involved in Alzheimer's disease (AD). Our results demonstrate that antibodies suitable for intracellular expression are present in natural repertoires, which have been incorporated into phage libraries,[14,15] and can be readily isolated with IACT, avoiding the need for any rational mutation strategy[16–18] or molecular evolution approaches.[10,19] Moreover, we found that ICAbs selected by IACT are characterized by a set of conserved residues that may define the subset of antibodies that function efficiently inside cells. This intracellular consensus sequence (ICS) may allow the design of improved antibody libraries biased towards ICAbs.

Results

The intracellular antibody capture technology

The yeast two-hybrid strategy[20] can be used to isolate intracellular binders from sets of scFv that bind antigen *in vitro*.[11] We used IACT to undertake the *de novo* selection of ICAbs against a variety of different proteins of interest, including the human neuronal MAP protein TAU. TAU undergoes a pathological self-assembly into neurofibrillary tangles in the brain of AD patients,[21] whose mechanisms *in vivo* are unclear and intracellular antibodies may help in addressing these mechanisms. Figure 1 illustrates the overall design of the selection process that leads from a phage antibody library to a validated ICAb, expressed in mammalian cells. The chosen bait protein (i.e. TAU) is first used to screen *in vitro* a library of scFv fragments displayed on phage[22] to generate a population of phages enriched for antigen-specific scFv. This *in vitro* panning step 1 (Figure 1) is needed because of the difference between the complexity of the antibody phage library and the transformation efficiency in yeast. We compared the results with one or two panning cycles *in vitro*, after which the antigen-enriched polyclonal population of scFv fragments is expressed as a ''prey'' in the yeast antibody-antigen interaction assay[23] and challenged with the TAU antigen bait (step 2, Figure 1). The recovered scFv are subsequently functionally assessed in mammalian cells (step 3, Figure 1).

To isolate anti-TAU ICAbs using IACT, TAU fragment 151-421, which encompasses two proline-rich regions and the microtubule binding repeats,[24] was fused to maltose-binding protein (TAU-MBP), and used for two successive rounds of *in vitro* panning (step 1, Figure 1) with a non-immune library of human scFv fragments.[22] The diversity of the enriched first and second-cycle libraries was assessed by PCR fingerprinting and sequence analysis of randomly isolated scFv fragments ($N = 100$ for each cycle). After the first cycle, 90% of the scFv fragments were different, while after the

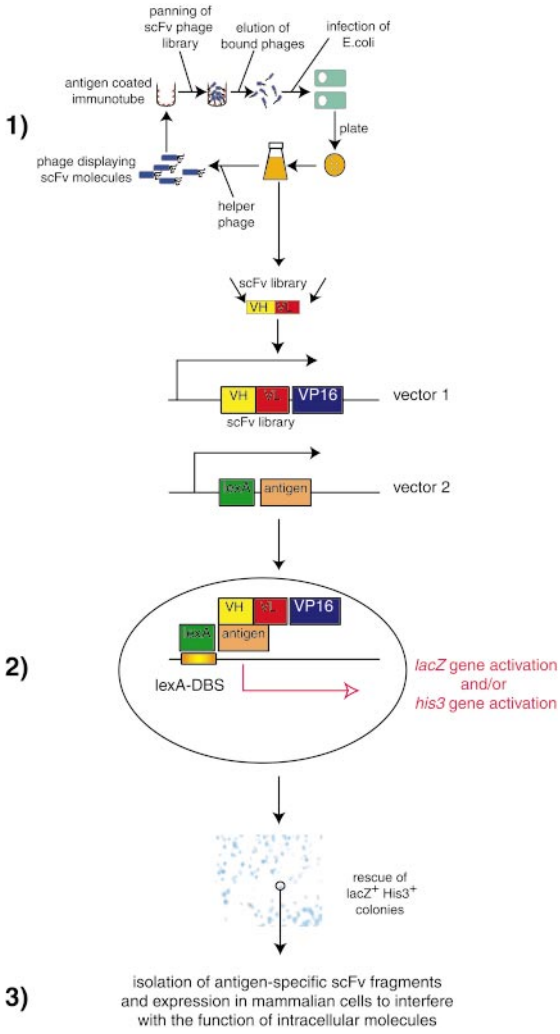

Figure 1. A schematic view of the intracellular antibody capture technology (IACT). It involves a combination of the yeast two-hybrid technology and the use of a phage-display library for the selection of antibodies. The preselection step by using phage display technology allows increasing the percentage of putative candidate scFvs in the input library. The yeast two-hybrid approach is adapted to isolate scFv-antigen interaction pairs under conditions of intracellular expression. After the *in vivo* selection, positive clones are isolated and scFvs are used for *in vitro* and *in vivo* applications.

second cycle only 13/100 different scFv fragments were found (Table 1). TAU-reactive phages were revealed by phage ELISA: three and nine scFv fragments out of 96 randomly selected clones were TAU-positive, after one and two panning cycles, respectively (Table 1) and did not bind MBP or other proteins (Figure 2(a)). The bulk DNA encoding for the first and the second round polyclonal scFv-anti-TAU fragments was subcloned into the prey vector VP16AD, yielding two libraries (anti-TAU/VP16AD-I and anti-TAU/VP16AD-II), consisting of 2.2×10^6 and of 6×10^4 clones, respectively. These sub-libraries were challenged in yeast

Table 1. Results of the *in vitro* and *in vivo* selections

	Diversity of the polyclonal anti-TAU library	ELISA screening	No. of positive interactions after 1 IACT screening	No. of different scFvs	No. of positive interactions after 2 IACT screenings
Round 1	90/100	3/96	$\sim 10^4$	31/100	17/31
Round 2	13/100	9/96	$\sim 10^5$	5/100	3/5

The number of positive clones and the number of monoreactive clones are shown.

against the TAU bait, fused to lexA DNA binding domain (lexA-151-422TAU). Transformants were selected for histidine prototropy and 10^5 yeast transformants were screened for LacZ activity.[23] Plasmid DNA was isolated from 100 strong blue colonies from each selection and analysed by fingerprinting, yielding 31 and five different scFv fragments from first and second round libraries, respectively (data not shown). These were individually tested in secondary yeast screening, yielding 17 and three anti-TAU ICAbs, respectively (Table 1, Figure 2(b)). It is noteworthy that the three anti-TAU/VP16AD-II were also found in the pool of 17 anti-TAU ICAbs selected from the first round library (data not shown). This demonstrates that performing only one round of enrichment *in vitro*, prior to the selection in yeast, yields a more complete repertoire of ICAbs. The specificity of the selected ICAbs was confirmed by co-transfection with other baits (data not shown). Four ELISA-positive anti-TAU fragments, randomly chosen from the enriched phage library among those that reacted more positively against TAU, failed to bind TAU bait (lexA-151-422TAU) in yeast, confirming that the *in vivo* selection procedure was indeed necessary (Figure 2(a) right).

Three anti-TAU ICAbs were expressed in *Escherichia coli* as periplasmic proteins and shown to recognize specifically full-length TAU and two deletion mutants (151-421TAUMBP and 151-422TAUMBP) (Figure 2(c)). ScFv fragments often show a propensity to aggregate[25] and aggregation of intracellularly expressed scFv fragments is often observed.[26] Gel filtration analysis of purified ICAbs showed, for all three scFvs, a single peak elution profile, corresponding to the monomeric form, as compared to the chromatogram of an aggregating scFv (Figure 2(d)). These data demonstrate the low aggregating propensity of the scFv fragments isolated by IACT. The affinity constants of the isolated scFv for TAU were evaluated by equilibrium saturation analysis/competition ELISA and by surface plasmon resonance and found to be in the range between 100 nM and 350 nM (data not shown).

Functional assays of anti-TAU ICAbs in mammalian cells

The anti-TAU ICAbs were expressed in mammalian cells, as leaderless cytoplasmic proteins.[26–28] They show a diffuse intracellular staining, typical of soluble cytoplasmic proteins (Figure 3), unlike the doughnut-like pattern shown by aggregating scFv fragments (scFvαD11 in Figure 3). The ability of the anti-TAU ICAbs to interact with TAU in mammalian cells was verified by a re-location assay. The anti-TAU scFv fragments were equipped with a nuclear localization signal (NLS) and individually co-expressed in CHO cells together with the TAU antigen (Figure 4(a)). The soluble scFvR4[8] was used as a negative control. Sub-cellular localization studies showed that TAU has a diffuse cytoplasmic distribution when transfected alone (Figure 4(a), top left) whereas each NLS-tagged scFv fragment, when expressed in the absence of antigen, is found in the nucleus (Figure 4(a), top right). When TAU is co-expressed with the non-relevant NLS-scFvR4 (Figure 4(a), bottom right) it remains localized in the cytoplasm (red staining), while the scFvR4 is found in the nucleus (green staining). By contrast, a predominantly nuclear staining for TAU antigen was observed after co-expression with the anti-TAU scFv fragments (Figure 4(a), centre left, centre right, bottom left) indicating an NLS-scFv-mediated re-location of antigen. For quantification (Figure 4(b)), co-transfected cells were subdivided in four groups, according to the pattern of double staining observed (described in Figure 4(c)). The first three groups correspond to a successful re-location event, due to an intracellular antigen-antibody interaction, while the fourth group indicates an independent location of antigen and antibody, i.e. no interaction. As shown in Figure 4(c), all cells transfected with the anti-TAU ICAbs are classified in one of the first three groups, while all cells expressing the control ICAb R4 fall in the fourth group (no relocation). This demonstrates the ability of anti-TAU ICAbs to bind specifically the TAU antigen *in vivo* in mammalian cells.

Expression in neuronal cell lines showed that at least one of the anti-TAU ICAb inhibits neurotrophin-mediated neurite outgrowth[29] (unpublished results).

In vivo epitope mapping (IVEM) of anti-TAU ICAbs

The characterization of the epitopes recognized by the anti-TAU ICAbs, was determined by *in vivo* epitope mapping (IVEM) with IACT. A panel of deletion mutants of the TAU protein starting from Ile151 to various residues towards the C terminus

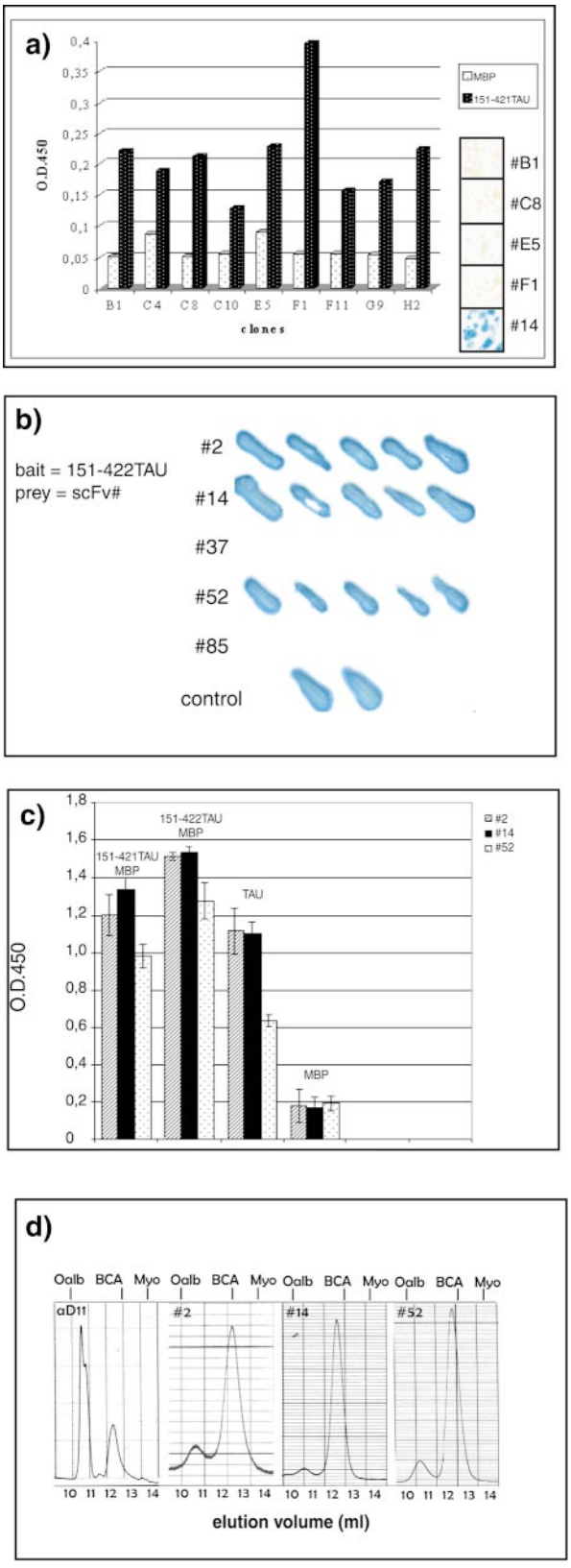

Figure 2. (a) ELISA signals of the phages isolated after two rounds of phage selection scheme. Antigens were coated at 10 μg/ml. Only nine scFvs out of 96 tested were specific for the antigen 151-421TAU-MBP protein. Specificity of binding was confirmed by ELISA signals on MBP protein. On the right, ELISA-positive anti-TAU no. B1, no. C8, no. E5 and no. F1 were shown not to react with LexA-151-422TAU bait in yeast. (b) Interaction of LexA-151-422TAU bait with the five independent scFv-fusion to VP16 AD-domain. L40 yeast strain was cotransformed with LexA-151-422TAU bait and the panel of the isolated scFv-VP16 plasmids. Only scFv no. 2, scFv no. 14 and scFv no. 52 were able to transactivate HIS3 and lacZ genes (rows 1, 2 and 4) while scFv no. 37 and no. 85 were not (rows 3 and 5). Interaction between scFvF8-VP16 and LexA-AMCVp41[11] was used as positive control (row 6). (c) ELISA of soluble fractions of *E. coli* cultures expressing scFv no. 2, scFv no. 14 and scFv no. 52 in the periplasmic space. Antigens were coated at 10 μg/ml. The ELISA signals were measured at A_{450nm}. (d) Analytical Superdex-75 gel filtration chromatograms of scFv no. 2, scFv no. 14, scFv no. 52 and the control aggregating fragment scFvαD11. Elution volumes and molecular mass marker proteins are indicated: ovalbumin (Oalb) (45 kDa), bovine carbonic anhydrase (BCA) (29 kDa), myosin (Myo) (17 kDa). Monomeric scFv is eluted at about 12 ml. 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.

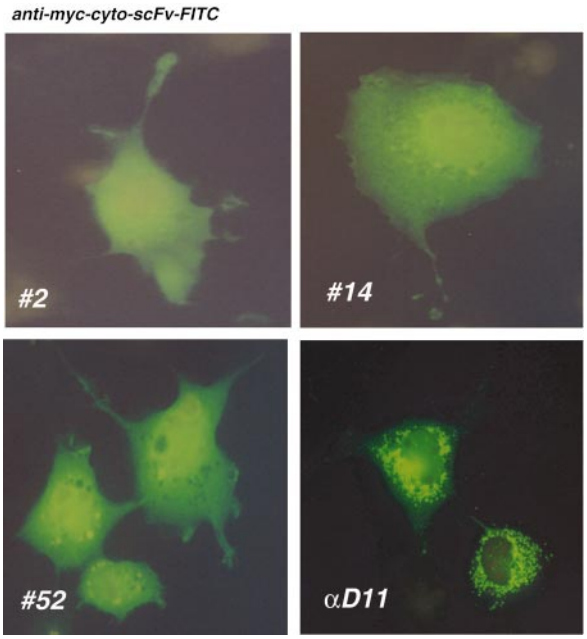

Figure 3. Immunofluorescence microscopy of the anti-151-422TAU scFv fragments transiently transfected in COS cells. Cyto-scFv no. 2, cyto-scFv no. 14 and cyto-scFv no. 52 and the control αD11 were visualized under a fluorescence microscope. Cells were reacted with the anti-*myc* tag antibody 9E10,[62] followed by incubation with an anti-FITC-conjugated anti-mouse (Vector).

of TAU (Figure 5(a)) was fused to LexA domain, yielding a set of TAU fragment baits. Each bait was challenged with each of the 17 anti-TAU ICAbs. Figure 5(b) shows the detailed results for three ICAbs (scFv fragments scFv no. 2, scFv no. 14 and scFv no. 52), showing, for instance, that

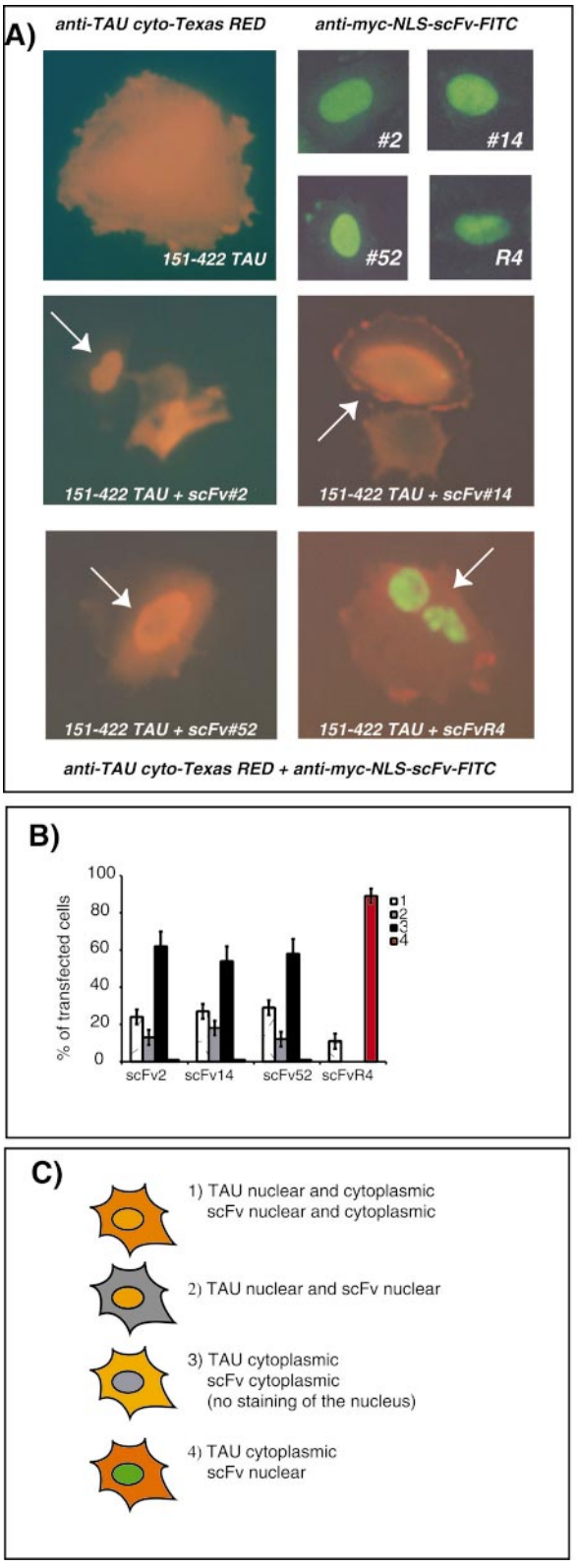

Figure 4. (a) Dual immunofluorescence analysis of the retargeting of 151-422TAU antigen in the presence of the different scFv fragments in CHO cells. Anti-TAU staining for the cytoplasmic (cyto) 151-422 TAU antigen was revealed with an anti-mouse Texas red-conjugated secondary antibody. Transfection of nuclear targeted (NLS) NLS-scFv no. 2, NLS-scFv no. 14, NLS-scFv no. 52 and NLS-scFvR4 were revealed with the anti-*myc* tag 9E10[62] as primary antibody and an anti-mouse FITC. Co-expression of cyto-151-422TAU antigen with NLS-scFv no. 2, with NLS-scFv no. 14, with NLS-scFv no. 52 and with NLS-scFvR4: all the NLS-scFv fragments were FITC-stained while the 151-422 TAU antigen was Texas red-stained. Transfected cells were visualized using a multiple wavelengths filter (Zeiss Filter Set 25). The arrows indicate the co-localization of NLS-anti-TAU scFv fragments in the nucleus with the retargeted cyto-TAU antigen. (b) and (c) Effect on retargeting of 151-422 TAU in CHO cells by scFv no. 2, scFv no. 14, scFv no. 52 and scFvR4. The staining pattern for TAU and the scFv fragments in the transfected cell population were subdivided into four classes, according to the type of double staining observed and the frequency of each group. Patterns (1), (2) and (3) are indicative of an interaction between TAU and the scFv.

scFv no. 2 binds to an epitope between T403 and S412. The overall picture for the whole panel of ICAbs shows that three main regions of TAU (I151-K274, N368-E391, D402-S412) are recognized by the selected ICAbs.

Sequence analysis of anti-TAU ICAbs

The sequences of ICAbs selected with IACT (IACT set) were compared to those of a group of randomly selected scFv fragments from the input library (control set) (17 and 16 sequences, respectively). All VH domains in the IACT set are VH III[30,31] while 13 VL domains are kappa I and four are kappa IV. Also in the control set most of VH sequences are VH III (only one was VH II), reflecting a bias in the input library. The VL in the control group are kappa I (ten sequences) and lambda (six sequences, mostly from subgroup IV). The average homology between individual members within the control set was 69 % for VH and 59 % for VL domains. The average homology within the IACT set was higher, 85 % for the VH and 77 % for the VL domains. Alignment of the VH and VL sequences of the IACT set (Figure 6) showed that, within this set, 76 amino acid residues in VH (including two from CDR 1, one from CDR 2 and one from CDR 3) and 48 in the VL domains are conserved. Conserved residues define an intracellular consensus sequence (ICS) for this set of antibodies (Figure 6).

A ''virtual consensus sequence'' can be defined from the antibody sequences in the Kabat database, generated from the most frequent amino acid at each position.[30,31] The degree of homology of the ICS to Kabat consensus sequences compiled from the Kabat database[30,31] was evaluated. All but two of the 76 conserved residues in the IACT VH set are equal to the most frequently found residue at this position, in the Kabat consensus sequence for human VH. The two positions that do not follow the Kabat consensus are occupied, nevertheless, by residues (Phe67 and Asn73) whose occurrence probability is only slightly lower than the highest

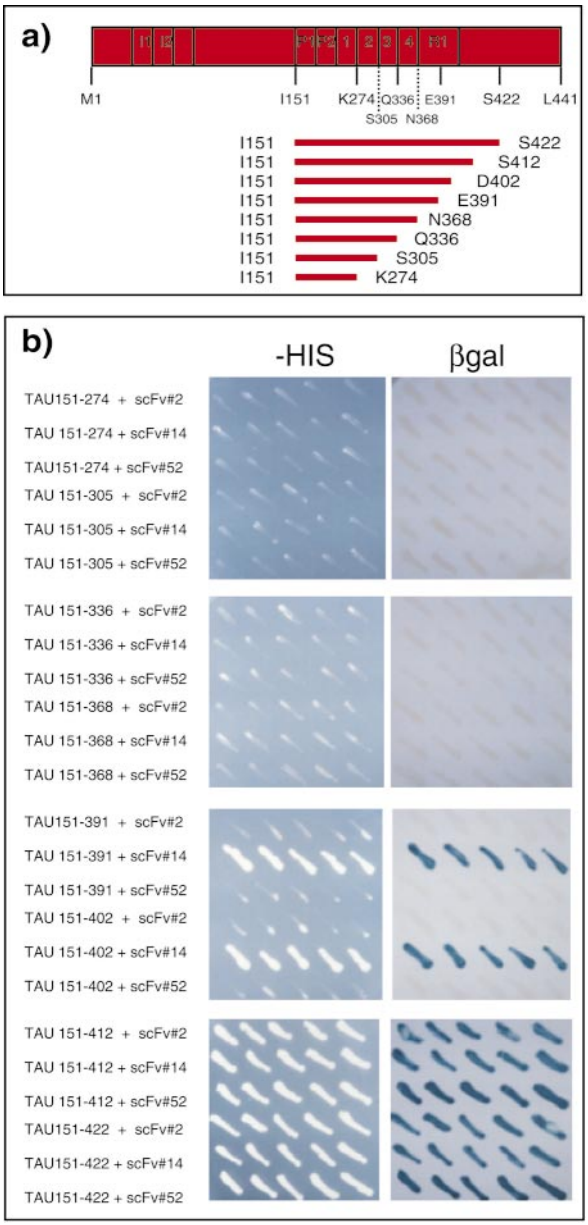

Figure 5. *In vivo* epitope mapping (IVEM). L40 yeast strain was cotransformed with LexA-antigen DNA-BD (panel of baits summarized in (a)) and anti-TAU scFv fusions with the VP16 transcriptional domain. (b) Yeast was streaked and grown on His-medium and scored for β-Gal activity.

one, in the Kabat consensus (Val in H67 and Thr in H73). Comparison with human VH III family, showed that only Gln in H1 is not as frequently found as Glu at this position, in the corresponding Kabat consensus set, while there is a perfect match at all the other positions. For the light chain, 47 residues out of the 48 conserved residues in the IACT VL set match the corresponding residues in Kabat consensus sequence while all the 48 conserved residues match the most frequently found in Kabat consensus for human VK.

Altogether, this analysis allows us to define an ICAb consensus (ICS), which is highly homologous to the consensus sequences for human VH and VL in the Kabat database and, in particular, to the Kabat consensus for human VHIII and VL K.

The same analysis was performed on a larger IACT database, with antibodies from different libraries and selected against different antigens, confirming the concept of a captured consensus sequence (unpublished results).

Discussion

Intracellular antibodies can be used to ablate protein function, with different modes of action.[1] Protein knock-out can be exploited for gene therapy, for target identification and validation in functional genomics, or to elicit antigen-specific cell killing.[32] Other applications include intracellular imaging[33,34] and diagnostics.[35,36] There is therefore the need for experimental strategies to isolate antibodies that are suitable for intracellular expression. Indeed, not all antibodies tolerate the reducing conditions of the cell cytoplasm.[7,26] To overcome these limitations we proposed that intracellular antibodies could be selected on the basis of their ability to bind antigen intracellularly.[11] Building on that proof of principle, we now provide evidence for the *de novo* selection of a panel of intracellular antibodies against a protein antigen involved in human AD.

Our results demonstrate that a large number of ICAbs against a single protein antigen are indeed present in large repertoires and that validated ICAbs can be readily and efficiently isolated and used with no need of individual optimization or engineering. We have now been able to select validated ICAbs against a number of different proteins (M.V. and A.C., unpublished results).

Sequence analysis demonstrated that IACT selection yielded sequences that are highly homologous to the consensus sequences for human VH and VL defined from the Kabat database. The latter are virtual consensus sequences, in that they are generated from the most frequent amino acid at each position. IACT leads to the selection of a sub-population of antibodies that are naturally present, albeit at low frequency, in antibody repertoires, and that are more effective in an intracellular environment. In particular, our results show that a combination of VH III and V K frameworks is likely to be more effective in this cellular environment.

The number of conserved residues in the IACT set defines an intracellular consensus sequence (ICS). As the database on ICAb sequences selected with IACT grows, the number of the ICS residues might decrease, converging to a minimal ICS (G.S., M.V., A.M. and A.C., unpublished results). Our results support the view that the overall stability of an antibody domain is contributed by a widespread set of stabilizing interactions between resi-

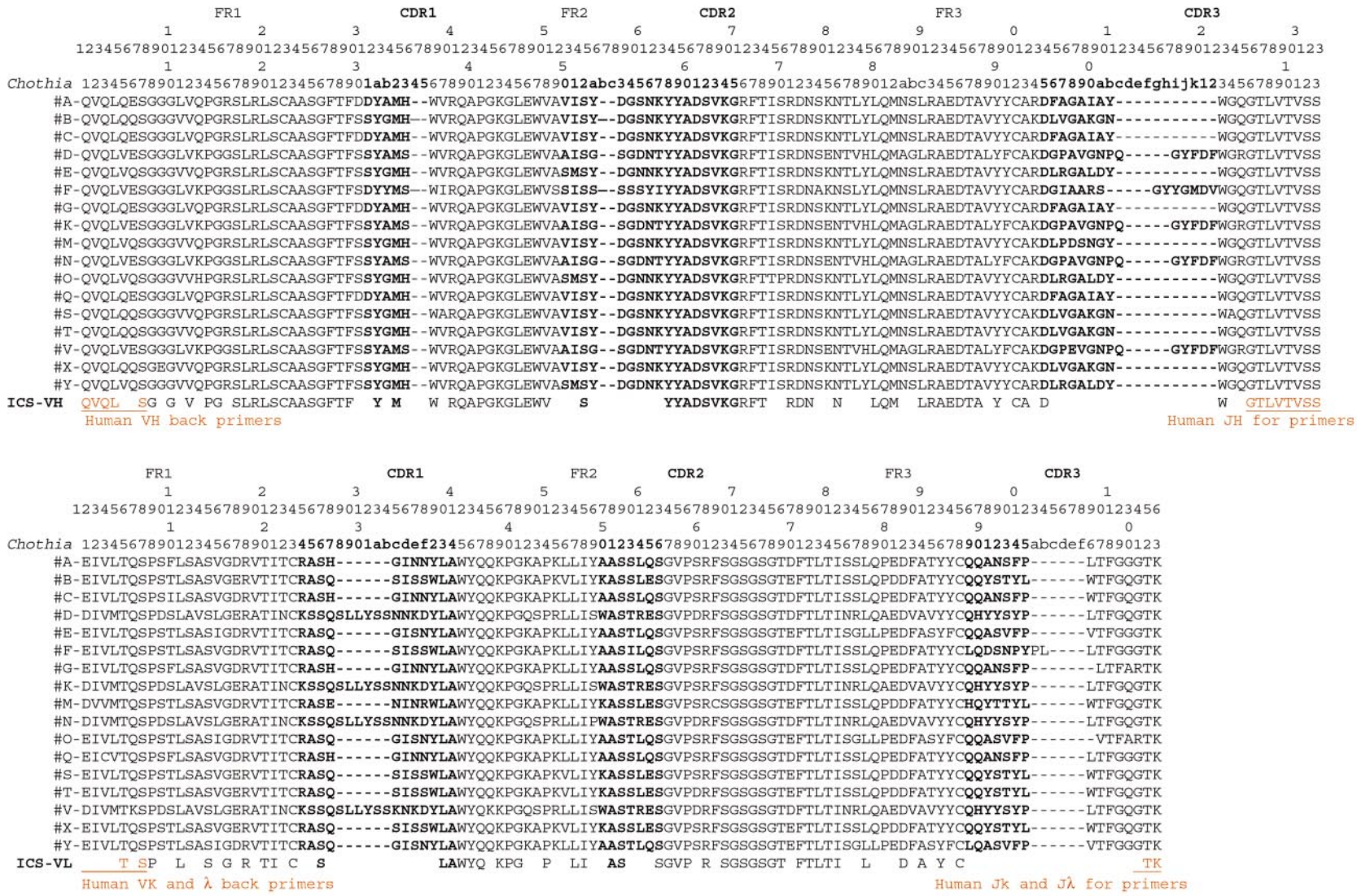

Figure 6. Sequence alignments of the VH and VL domains of the anti-TAU scFvs. The hypervariable regions (bold) are defined as by Chothia and Lesk.[63] The conserved residues (intracellular consensus sequence, ICS) for VH and VL are listed below each set of sequences. Primers for amplifications of V domains are labelled in red.

dues. Consensus sequence engineering has been previously shown to lead to improved ICAbs on a case-by-case basis.[17,37] Our results show that IACT naturally provides a robust, efficient and generic biological consensus sequence selection method, leading to a sort of ''captured consensus''. It should be possible to construct input libraries based on captured consensus. This will allow us to bring the input library down to a size compatible with its direct transformation in yeast, while maintaining its full diversity, further facilitating and accelerating the selection of ICAbs to target proteins of interest.

The large panel of anti-TAU ICAbs provides a useful tool to study TAU function in neurodegenerating neurons and brains.

For many applications, it is desirable to have antibodies that bind to a given epitope of an antigen. Different approaches are available, all involving epitope mapping *in vitro*.[38–41] We describe here a rapid *in vivo* epitope mapping method (IVEM), which may become the method of choice for mapping the epitopes of ICAbs selected with IACT, and should greatly accelerate the detection of critical epitopes in high throughput screens.

IACT could be used, in conjunction with the surface display of cDNA products on *E. coli*[42] or on lambda phage,[43,44] to derive ICAbs directly from gene sequences, with minimal handling of the corresponding proteins. IACT provides a direct link to go from phage display antibody libraries[45] to gene function. The preselection of phage antibody libraries on antigen arrays[46,47] may represent one viable possibility to provide a diverse but enriched input library to yeast.

The need for technologies that allow studies of protein function in a highly parallel and high throughput manner suggests our protein knockout technology as a rapid, low-cost and simple way to deal with the functional genomics bottlenecks. The ability of IACT to isolate validated ICAbs, to be used for the validation of therapeutic targets, will help in the cell-wide analysis of protein networks and provides a new enabling tool for functional genomics and global proteomics.

Materials and Methods

Plasmids

Construction was performed according to standard techniques.[48]

TAU deletion mutants (151-274, 151-305, 151-336, 151-368, 151-391, 151-402, 151-412, 151-422) were cloned into the plasmid pBTM116 between *Eco*RI/*Bam*HI fragments.[24,49]

TAU 151-421MBP: the DNA fragment 151-421 TAU was PCR amplified from TAU 40/pSG5[49] using appropriate primers. The *Eco*RI/*Sal*I-digested fragment was cloned into pMAL-c2 (NEB) down-stream from the malE gene.[50–52] TAU 151-422MBP: the TAU 151-422 *Eco*RI/*Bam*HI[24] fragment was subcloned into *Eco*RI/*Bam*HI sites of pMAL-c2.

ScFv fragments were subcloned into the plasmids pUC119CAT (kind gift from T.H. Rabbitts) between *Sfi*I/*Not*I restriction sites and between *Nco*I/*Not*I restriction sites of scFvexpress plasmids[27] All clones were sequenced, using the Epicentre Sequitherm Excel II kit (Alsbyte, Mill Valley, CA), with a Li-Cor 4000L automatic sequencer (Lincoln, NE).

Protein productions

TAU protein was purified from BL21(DE3) bacterial cells (kind gift from M. Novak) as described.[53] The MBP-fusion proteins were purified by one-step affinity purification for MBP as described.[52]

Selection of phage antibody library

A non-immune phage antibody library[22] was selected as described[54] with TAU 151-421MBP protein-coated immunotubes (Nunc, Rochester, NY) (10 mg/ml overnight). After the first and the second cycle of selection, 96 scFv fragments from individual colonies were identified by *Bst*NI fingerprinting. DNA fingerprinting and sequencing of 20 clones from each library confirmed that the diversity was maintained. The same clones were used in phage ELISA.[54]

Construction of an activation domain-fusion anti-421MBP scFv library

Phagemid DNA from the first and the second round phage library was extracted (Qiagen midiprep kit) and scFv fragment DNA subcloned into *Sfi*I/*Not*I-cut pVP16.[55] The ligation mix was electroporated into electrocompetent DH5αF′ (Gibco BRL, Rockville, MD) and plated on LB/ampicillin (100 μg/ml) plates to obtain the AD-libraries.

Yeast strain and protocols

The AD-fusion anti-151-421TAU scFv libraries were transformed in L40 *Saccharomyces cerevisiae*[56] containing TAU 151-422/BTM116 bait plasmid following a modification of published methods:[23] 90% of the clones grown in the absence of histidine gave a strong reaction in the β-Gal assay; 100 individual AD-fusion scFv plasmids were isolated from segregated yeast cells as described.[23] Plasmids were rescued in DH5αF′ *E. coli* and scFv inserts were identified by PCR-*Bst*NI fingerprinting and by sequencing as described.[54] The isolated AD-fusion scFv plasmids were co-transformed with the LexA-151-422TAU antigen bait vector into L40 yeast strain by a lithium acetate transformation protocol[57–59] and preselected by using auxotrophic markers and the X-Gal lift assay.

Expression and purification of scFv fragments

The scFvs were expressed from the plasmid pUC119-CAT in HB2151 *E. coli*, grown at 30 °C for four hours after induction with 0.5 mM IPTG and purified using the Ni-NTA kit (Qiagen, Valencia, CA). After verification of scFv purity by SDS-PAGE, scFv concentrations were quantified using the bicinchoninic acid kit (Pierce).

Analytical gel filtration

Gel filtration was carried out on a Superdex 75 (HR 10/30) column (Amersham Pharmacia Biotech, UK) in PBS. The 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.

Cell lines, transfection and immunofluorescence analysis

COS simian fibroblasts were grown in DMEM with 10% (v/v) fetal bovine serum and high glucose. Cells were transiently transfected by the DEAE-dextran method[49] and analysed 48 hours after the transfection. Anti-TAU MN7.51 hybridoma supernatant[60] was used at a 1:50 dilution. mAb anti-*myc* antibody 9E10[61] and polyclonal anti-*myc* (Clontech) were used at 1:50 and 1:40, respectively. Texas red-conjugated anti-mouse (1:50 dilution, Vector), fluorescein goat anti-mouse conjugate (1:50 dilution, Vector) or anti-rabbit immunoglobulins fluorescein-conjugate (1:50 dilution, Vector) were used as secondary antibodies. Nuclei were stained with the fluorescent dye DAPI (Boehringer Mannheim).

CHO (Chinese Hamster Ovary) cells were grown in DMEM with 10% fetal bovine serum, high glucose and proline (17.2 mg/l final concentration). CHO were transiently transfected by the FuGENE-6 transfection reagent (Roche) method according to the manufacturer's instructions and analysed 30-48 hours after the transfection.

Sequence analysis

Sixteen scFvs from the input library and 25 anti-TAU scFv selected with IACT were sequenced, using the Epicentre Sequitherm Excel II kit (Alsbyte, Mill Valley, CA), on a Li-Cor 4000L automatic sequencer (Lincoln, NE). Sequence alignments and analysis were carried out as described by Kabat.[30,62]

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