Intracellular Stability of Anti-caspase-3 Intrabodies Determines Efficacy in Retargeting the Antigen

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Although intracellular antibodies (intrabodies) are being explored as putative therapeutic and research reagents, little is known about the principles that dictate the efficacy of these molecules. In our efforts to address this issue, we generated a panel of five intrabodies, directed against catalytically inactive murine caspase-3, by screening single-chain antibody (Fv) phage display libraries. Here we determined criteria that single-chain Fv fragments must fulfill to act as efficient intrabodies. The affinities of these intrabodies, as measured by surface plasmon resonance, varied ~5-fold (50–250 nM). Despite their substantial sequence similarity, only two of the five intrabodies were able to significantly accumulate intracellularly. These disparities in intracellular expression levels were reflected by differences in the stability of the purified protein species when analyzed by urea denaturation studies. We observed varied efficiencies in retargeting the antigen murine caspase-3, from the cytosol to the nucleus, mediated by intrabodies tagged with an SV40 nuclear localization signal. Our results demonstrate that the intrinsic stability of the intrabody, rather than its affinity for the antigen, dictates its intracellular efficacy.

The design, cloning, expression, and use of single-chain antibodies (scFv)† have now become routine in protein engineering. These moieties, although monovalent in comparison with their bivalent precursor IgG, do not suffer from the need to produce and assemble multichain oligomers like other members of the immunoglobulin family. scFv fragments have been shown to display similar (although not identical) kinetic and thermodynamic properties as Fab fragments of whole IgG (1). In recent years, filamentous phage display libraries of scFv molecules derived from the human IgG repertoire have become increasingly available (2–4). It is now possible to generate a scFv molecule against a target within a few weeks. However, since these libraries are usually generated from non-immunized sources and hence have not undergone affinity maturation, the isolation of high (subnanomolar equilibrium constant for dissociation) affinity scFv fragments from these selections is rare.

Among the myriad applications of these molecules, the use of scFv fragments as intrabodies (intracellularly expressed antibodies) has received some attention recently. The aim of such attempts has been to neutralize the function of endogenous target proteins, using intrabodies, by several methods (5–7). Among these, the misdirected localization of the target to another subcellular region features as the most popular methodology employed. These initial promising results suggest potential applications for intrabodies in functional genomics (8) and gene therapy (9, 10). The ability of intrabodies to generate phenotypic knockouts in vivo is now being explored in several organisms (11, 12).

The aim of this study was to determine the biochemical principles involved in the generation of effective intrabodies. We selected apoptosis (programmed cell death) as our model system, in which specifically caspase-3 was chosen as the target. Caspase-3 (CPP32/CASP3) is a member of a family of thiol proteases involved in programmed cell death (13–17). These proteases are initially synthesized as inactive proenzymes that are proteolytically activated by upstream activators upon apoptotic stimulation. Caspase-3 has been implicated as a downstream component of the apoptotic machinery and is involved in the proteolysis of several cellular components. Among the several moieties involved in apoptosis, at the time we initiated the project, it was most facile to generate caspase-3 protein in bulk quantities as antigen for the selection of scFv fragments. Other more attractive targets such as caspase-8 were difficult to overexpress in Escherichia coli due to loss of protein through aggregation.

A panel of scFv fragments was isolated from a phage display library directed toward the antigen murine caspase-3 (mCASP3). These scFv fragments were tested for properties such as affinity for the antigen, protein stability, and intracellular accumulation to determine characteristics that define an efficient intrabody. In conjunction with these studies, the scFv fragments fused with an SV40 nuclear localization signal (NLS) were utilized to retarget antigen from the cytoplasm to the nucleus in an effort to correlate intracellular behavior with biochemical parameters. Our data suggest that the stability of an intrabody is the most significant attribute, more so than affinity for the antigen, in dictating its effectiveness intracellularly. The intracellular stability of an intrabody was also found to correlate with the stability of the purified protein, as measured by urea denaturation under standard and reducing conditions. To our knowledge, this is the largest panel of scFv fragments against a common antigen that have been tested for intracellular stability and efficacy as intrabodies thus far.

MATERIALS AND METHODS

Cell Lines and Transfections—293T transformed human embryonic kidney cells and BHK-21 baby hamster kidney cells were grown...
in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, and gentamicin (all from Life Technologies, Inc.). These cells were grown at 37°C in 5% CO2.

For Western blot and co-immunoprecipitation studies, 1 × 106 293T cells were plated on 100-mm dishes and transfected with 10 μg of DNA using 30 μl of Fugene-6 (Roche Molecular Biochemicals). Green fluorescent protein-pcDNA3.1 was used as a control, and transfection efficiencies were determined by fluorescence-activated cell sorter analysis of trypsinized 293T cells.

3 × 108 BHK-21 cells were coated onto coverslips in six-well dishes. 2 μg of transfected DNA was transiently transfected into these cells using 4 μl of LipofectAMINE and 6 μl of Plus reagents (Life Technologies, Inc.). These cells were utilized for immunofluorescence investigations.

**Heterologous Expression of mCASP3 in E. coli**—The gene encoding mCASP3 was amplified from a mouse liver library (Stratagene) using gene-specific primers designed from published sequences (GenBank24/5 EBI Data Bank accession number U49929). The oligonucleotide primers 5′-CATGCCATGGAGAACAACAAAACC3′ and 5′-CGCCGATCCT-AGTGGTGCTTGGTGTTGCTGATCATCCTGGTGCTTGAT-TAGATAAATTACAGTTC-3′ were utilized to clone the mCASP3 gene into bacterial expression vector pET15b (Novagen). The oligonucleotide introduced a FLAG (DYKDDDDK) and a hexahistidine tag at the 3′-end of the mCASP3 coding sequence. The following primers were used to generate inactive protease (by converting the active site cysteine to serine) (C163S): 5′-ATTCCAGGCCACGCGGCGTACG and GTACCAGGCCGTGCTGCTAAT-3′. Protein expression was optimized by selecting the highest expressing bacterial colonies, which were then grown in large scale (6 × 1 l) at 37°C until mid-log phase (A600 ~ 0.4). These were then induced by 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) for 4 h at 30°C. The bacterial pellet was collected by centrifugation and lysed by sonication (5 × 1 min) in ~50 ml of lysis buffer (25 mM HEPES (pH 7.5), 2 mM DTT, 200 mM sodium chloride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM Pefabloc-sc). Cellular debris was removed by centrifugation (15,000 rpm, Sorvall SS-34 rotor), and the cleared lysate was diluted in chilled lysis buffer to 200 ml. 5 ml of Ni2+-nitrilotriacetic acid (NTA) Superflow resin (QIAGEN Inc.) was added to the lysate and incubated at 4°C with shaking overnight. The resin was then collected by centrifugation (3000 rpm, Beckman GS-6) and washed three times with 50 ml of wash buffer (25 mM HEPES (pH 7.5), 2 mM DTT, 200 mM sodium chloride, 0.5 mM imidazole, and 0.1% CHAPS). The resin was then packed in a column, and the protein was eluted with a salt gradient (0–1 M sodium chloride in 25 mM Tris (pH 8.0), 2 mM DTT, 200 mM sodium chloride, 0.5 mM imidazole, and 0.1% CHAPS). The resin was then washed with 15 ml of elution buffer (25 mM HEPES (pH 7.5), 2 mM DTT, 200 mM sodium chloride, 300 mM imidazole, and 0.1% CHAPS). The eluate was dialysed overnight against 25 mM Tris (pH 7.5) and then concentrated using the Amicon Ultra-4 centrifugal filter device. The semi-pure protein (see Fig. 1A) was then loaded onto a pre-packed Mono-Q column (Hi-Trap, Amersham Pharmacia Biotech), washed, and eluted with a salt gradient (0–0.5 M sodium chloride in 25 mM Tris (pH 8.0) and 2 mM DTT).

The coding sequences for the anti-C163S-mCASP3 scFv fragments were cloned into the Myl and Nos1 sites of expression vector pUC119. This resulted in the **Protein Stabilization by Urea Denaturation**—Urea stock solutions were prepared according to details provided elsewhere (19). Briefly, 60 g of ultrapure urea (Fisher) was dissolved in a total volume of ~100 ml of PBS. The final concentration of the solution was determined by refractive index measurements. Typically, 40 μg/ml scFv solutions were made in varying concentrations of urea, 100 mM PBS, and 100 mM sodium chloride and incubated at room temperature overnight. Protein denaturation was followed by measuring the change in the intrinsic fluorescence emission spectra (at 320 nm) after excitation at 278 nm on a PerkinElmer Life Sciences LS 50B spectrofluorometer. The data were then normalized and fitted to simple two-state denaturation curves as described before (20). The data were not used to compute free energies because our main purpose in doing the experiment was to obtain a qualitative ranking of the scFv fragments according to their restriction enzyme mapping pattern. Representatives from these groups were further analyzed by DNA sequencing.

**Western Blotting and Co-immunoprecipitations**—48 h post-transfection, the medium was aspirated from the dishes, and the cells were washed with 5 ml of cold PBS. Cells were lysed in 1 ml of immunoprecipitation buffer (0.25% Nonidet P-40, 100 mM sodium chloride, and 50 mM Tris (pH 8.0)) supplemented with protease inhibitors (Complete medium, Roche Molecular Biochemicals) at 4°C for 90 min. The lysate was cleared by centrifugation and incubated with 6 μg of anti-FLAG antibody (M2, Sigma) and 50 μl of protein A-Sepharose (Amersham Pharmacia Biotech) slurry overnight with constant mixing at 4°C. The Sepharose beads were washed twice with immunoprecipitation buffer before being boiled in 1.5% SDS gel loading buffer. The proteins were separated by gel electrophoresis on 4–12% NuPAGE gels (Novex) and blotted onto nitrocellulose membranes by standard techniques. Western blot analysis was undertaken with a 1:4000 dilution of anti-Myc antibody (9E10) and a 1:1500 dilution of anti-FLAG antibody (M2). Horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) at a 1:3000–5000 dilution were utilized in conjunction with the ECL detection kit (Amersham Pharmacia Biotech) to visualize the results.

A mixture of proteasome inhibitors was made with 10 mM benzoxycarboxyl-Tyr-Val-Leu-arg (Boc-DTT) and 10 mM benzoxycarboxyl-Leu-Leu-leucinal (Calbiochem). A final concentration of 10 μM each was mixed with the growth medium and added to 293T cells transiently transfected with anti-mCASP3 scFv fragments (24 h post-transfection) for 24 h. The cells were then harvested, and Western blot analysis with anti-Myc antibody (9E10) was undertaken to determine the relative intracellular levels of the scFv fragments.
rabbit anti-Myc antibody (U. S. Biochemical Corp.) and 20 μg/ml mouse anti-FLAG antibody (Sigma) in PBS and 0.1% BSA (PBS/BSA) and incubated in a humidifying chamber at 37 °C for 1 h. Excess antibodies were washed out by washing the coverslips with PBS/BSA three times for 10 min each. The cells were stained with 30 μg/ml anti-rabbit FITC and anti-mouse Texas Red antibodies (Jackson ImmunoResearch Laboratories, Inc.) diluted in PBS/BSA at 37 °C for 30 min. Finally, the cells were washed with PBS/BSA (3 × 10 min) and mounted in 50% PBS and 50% glycerol with 25 mg/ml 1,4-diazabicyclo[2.2.2]octane (Aldrich). The cells were viewed on a Nikon FXA microscope fitted with a CCD camera.

**RESULTS**

Expression and Characterization of mCASP3—A catalytically inactive mutant of murine caspase-3 (the active-site cysteine was converted to serine, C163S-mCASP3) was engineered to prevent any proteolytic degradation of phage display scFv fragments during the screening process. Purification of heterologously expressed C163S-mCASP3 was performed on Ni²⁺-NTA affinity resin, followed by a Mono-Q column (Fig. 1). Fig. 1A (third and seventh lanes) shows the precipitate formed in the lysate and eluate, respectively. Typical yields of the final purified C163S-mCASP3 protein were in the vicinity of 3 mg/liter of medium. This yield may have been increased if precipitation of protein was minimized after elution from the Ni²⁺-NTA column. The final purity of the protein was determined by silver staining SDS-polyacrylamide gel (Fig. 1B). The observed band at ~62 kDa is the dimer of protein, as verified by N-terminal sequencing of the first 12 amino acids (data not shown). It is unclear to us why this exists in the presence of a reducing/denaturing environment. The molecular mass (as determined by electrospray mass spectrometry) of the protein was similar to that expected from the amino acid sequence (33,284 Da versus the expected mass of 33,275.6 Da), >90% of the protein existed as a dimer in solution as determined by gel filtration chromatography (data not shown).

scFv Fragments Isolated by Screening Phage Display Libraries—Anti-C163S-mCASP3 scFv fragments were obtained by utilizing the full-length protein as a target in multiple rounds of screening against phage display scFv libraries. Input titers for each of the phage libraries were considerably high (≥10¹² plaque-forming units/ml). After the first round of selections, the output titers dropped significantly (10⁵ plaque-forming units/ml). These phage were grown, and subsequent input phage titers were ~10¹¹ plaque-forming units/ml. As expected, the output titers for the second round were higher (~20-fold) than those obtained for the first round.

We decided to limit the selections to only two rounds in order to obtain clonal diversity in the positives scFv fragments yielded by the libraries. After a preliminary soluble ELISA screen of the outputs from the two libraries, it was determined that only the combined spleen library yielded binders (Fig. 2A). ~600 clones (six 96-well plates) from this library were grown, induced overnight, and osmotically shocked to obtain crude scFv. These scFv fragments were tested for antigen affinity by soluble ELISAs utilizing the IGEN. ELISA yielded 70 clones that displayed moderate to strong affinity for the antigen C163S-mCASP3 (electrochemiluminescence signal >10⁴). The specificity of these clones was tested using other caspases (mCASP2 and mCASP8) as antigen in similar soluble ELISAs. The results demonstrated that the isolated scFv fragments were specific for their antigen, C163S-mCASP3, with negligible cross-reactivity against the other caspase family members (data not shown).

The diversity in these clones was determined by restriction enzyme mapping using BstNI (Fig. 2B). Clones that displayed similarly sized bands after the restriction enzyme digest were grouped together into clonal groups (total of eight). Representative members from these groups were then sequenced. As expected, members within a clonal group (as defined by the BstNI digest) had identical DNA sequences. However, upon DNA sequencing and subsequent translation into polypeptide sequences, three clonal groups had identical amino acid sequences compared with other groups (of the remaining five) and were therefore grouped accordingly. Representative members from the five distinct polypeptide groups of anti-C163S-mCASP3 scFv fragments were then isolated and cloned into eukaryotic and prokaryotic expression vectors.

**Characterization of the Anti-C163S-mCASP3 scFv Fragments**—The scFv fragments were constructed in the V₅H-SS-
(GGGS)$_3$V$_L$ format (where V$_H$ and V$_L$ indicate variable regions of heavy and light chains, respectively). A protein sequence alignment for the five scFv fragments selected is shown in Fig. 3. The polypeptide sequences of the five scFv fragments are 50–70% identical, with the constant regions and the light chains displaying maximal similarity between the clones. As expected, the highest diversity between the sequences lies in the complementarity-determining regions (specifically regions H3 and L3). The five clonally distinct scFv fragments were subcloned and overexpressed in *E. coli* with subsequent purification to yield near-homogenous protein solutions (Fig. 4). Fractions 12 and 13 were pooled and analyzed by electrospray mass spectrometry to verify the molecular mass and identity of the purified protein (data not shown). The oligomeric state of these scFv fragments was determined by gel filtration chromatography to be predominantly monomeric (data not shown). The identity and cleavage site of the N-terminal signal sequence of all five scFv fragments were determined by N-terminal sequencing.

The affinity of these scFv fragments for the antigen C163S-mCASp3 was determined by surface plasmon resonance (Fig. 5). All scFv fragments were tested for nonspecific binding using mCASp2, a non-reacting antigen, as analyte. The scFv fragments maintained affinity for the antigen C163S-mCASp3 tagged with an alternative epitope tag (the influenza hemagglutinin epitope YPYDVPDYA) instead of FLAG to ensure specific binding between the moiety. Both epitope tags on scFv (Myc and His) were utilized to capture on the CM5 chip to eliminate the possibility of nonspecific binding between components of the reaction. Only the equilibrium constants for dissociation were determined by the BIAcore analysis because obtaining kinetic information was precluded by complex kinetic behavior observed for some of the scFv-antigen complexes.

The affinities for the antigen varied from 50 to 250 nM (Table I). The $K_D$ for 3C1-A2 could only be estimated because slow association kinetics and only semi-equilibrium conditions prevented an accurate measurement for this scFv fragment. The value of $\sim 100$ nM is an upper limit since measurements of RU for injections of lower analyte concentrations (200 nM and below) were underestimated compared with those expected at equilibrium. Attempts to obtain kinetic/equilibrium data for the system in reverse (i.e. capturing antigen with anti-FLAG antibody and using scFv fragments as analytes) were unsuccessful. We hypothesize that the epitopes for these scFv fragments are inaccessible in this experimental system, or the capture of the antigen affects the secondary, tertiary, and/or quaternary conformation of the protein.
were fitted to a single interaction model that allows for the determination of the equilibrium constants for dissociation of the anti-C163S-mCASP3 scFv and C163S-mCASP3 (0, 10, 40, 70, 100, 200, 400, 500, and 1000 nM; B, plot of ΔRU versus concentration of caspase-3 analyte. The data were fitted to a single interaction model that allows for the determination of $K_D$.

**TABLE I**

| scFv     | $K_D$ (nM) |
|----------|------------|
| 3C1-A2   | $< 10^9$   |
| 3C1-B1   | $51 \pm 2$ |
| 3C1-C4   | $237 \pm 26$ |
| 3C1-D3   | $252 \pm 15$ |
| 3C3-C1   | $66 \pm 20$ |

*See “Results” for an explanation.*

**Stability of the scFv Fragments**—Western blotting of transiently transfected cell lysates was performed to initially determine the intracellular expression levels of the anti-C163S-mCASP3 scFv fragments. Fig. 6A demonstrates that 3C1-A2 (first lane) was clearly the most intracellularly abundant among the five scFv fragments. The rationale for the lack of intracellular accumulation of the other four scFv fragments was not immediately apparent because of the absence of consensus PEST sequences (22) (data not shown) and the substantial sequence similarity between the scFv polypeptides. Inhibitors were utilized to gauge the role of proteasome-mediated degradation of these intracellular scFv fragments. Upon soaking 293T cells with a mixture of these inhibitors, we were able to modestly increase the observable intracellular concentration of the four previously less abundant scFv fragments (Fig. 6, A and B, compare second through fifth lanes). The pcDNA3.1 lane in Fig. 6B (sixth lane) is the vector-alone control.

Urea denaturation studies were undertaken to determine the stability of each scFv fragment. These studies were possible only with three of the five scFv fragments due to the lack of starting material for 3C1-B1 and 3C3-C1. In nonreducing environments, the midpoints of denaturation ($C_m$) for 3C1-A2 and 3C1-C4 were very similar and substantially greater than that of 3C1-D3 (Table II and Fig. 7A). These data agree with those obtained by Western blotting (Fig. 6A), where 3C1-A2 and 3C1-C4 were the only scFv fragments clearly detectable. However, the large discrepancy between the expression levels of the two scFv fragments is not evident from the two similar $C_m$ values under nonreducing conditions. It is more evident with the data obtained in reducing environments (in the presence of 20 mM DTT) (Fig. 7B). In the presence of reducing agents, the $C_m$ for 3C1-A2 was significantly higher than those for 3C1-C4 and 3C1-D3 (Table II).

**Intracellular Specificity of scFv 3C1-A2**—To test the specificity of the interaction between scFv 3C1-A2 and C163S-mCASP3, a co-immunoprecipitation experiment was conducted. 293T cells transiently cotransfected with the intrabodies (with C-terminal Myc tags) and C163S-mCASP3 (with a C-terminal FLAG tag) were lysed and immunoprecipitated with anti-FLAG antibody (M2). The components of the immune complex were separated by SDS-polyacrylamide gel electrophoresis and probed with anti-Myc antibody by Western blotting. The data demonstrate the specificity and affinity of 3C1-A2 for its target C163S-mCASP3 as shown by co-immunoprecipitation (Fig. 6C, first and second lanes). The experiment was done in duplicate and showed no detectable levels of C163S-mCASP3 in the co-immunoprecipitates obtained from cells cotransfected with the other scFv fragments (Fig. 6C, third through tenth lanes). The pcDNA3.1 lanes are the vector-alone controls. We expected to detect 3C1-C4 on the Western blot (Fig. 6C) and surmise that its intracellular levels precluded detection by the methodology employed here.

Immunofluorescence studies were performed to further characterize the intracellular scFv-antigen interaction. The caspase-3 precursor has been shown to have a mitochondrial and cytosolic subcellular distribution in non-apoptotic cells (23). To test the specificity and affinity of the interaction between scFv...


Table II

Assessment of the in vitro stability of three anti-C163S-mCASP3 scFv fragments by urea denaturation

| scFv       | Nonreducing Cₘ | Reducing Cₘ |
|------------|----------------|-------------|
| 3C1-A2     | 5.41 ± 0.02    | 3.25 ± 0.01 |
| 3C1-C4     | 5.20 ± 0.02    | 2.93 ± 0.02 |
| 3C1-D3     | 4.52 ± 0.02    | 2.83 ± 0.02 |

* Urea denaturation in the presence of 20 mM DTT.

Fig. 7. Urea denaturation of three anti-C163S-mCASP3 scFv fragments. •, 3C1-D3; ○, 3C1-C4; ●, 3C1-A2. A, purified scFv fragments (40 μg/ml) were incubated overnight at room temperature in the indicated concentrations of urea, fraction unfolded was determined by fluorescence at 345 nm (excitation at 275 nm). B, the same as A, except for the inclusion of 20 mM DTT in the urea solution. For further details, see "Materials and Methods."

3C1-A2 and C163S-mCASP3, an SV40 NLS (24) was fused to the C terminus of scFv. C163S-mCASP3 is predominantly a cytosolic protein (Texas Red channel (right panel) in Fig. 8A), and scFv 3C1-A2 resides in the nucleus, as expected (FITC channel (left panel) in Fig. 8B). Upon transiently transfecting both C163S-mCASP3 and 3C1-A2, we observed the subcellular retargeting of the antigen that can only be ascribed to scFv. Representative data are shown in Fig. 8C, where in the Texas Red channel (right panel), we observed the retargeting of cytosolic antigen into the nucleus. The middle panels in Fig. 8 show the bright-field images of the respective cells, in which the cytosolic and nuclear compartments are clearly visible. The data also demonstrate that an intrabody with an SV40 NLS resides in the nucleus versus staining in the cytoplasm, as visualized by Texas Red staining in the right panels. This hierarchy in re-localization efficiencies is mimicked by the level of intracellular accumulation of the individual scFv fragments as depicted by the green FITC staining in the left panels. Similar studies with scFv 3C1-B1 were less informative due to low expression levels of the intrabody.

Discussion

Panning phage display libraries has become a standard technique for the isolation of moderate affinity scFv fragments that recognize small molecule and macromolecular antigens (2–4). As might be expected, the diversity of the library dictates the ability to obtain multiple binders for antigens. In our experience, we found that the bone marrow-derived library yielded no anti-C163S-mCASP3 scFv fragments despite substantial screening of second, third, and fourth round selectants. This might be attributed to the lack of diversity in the light chain of scFv fragments displayed in this library. The affinity of the scFv fragments isolated (from the combined spleen library) ranged from 50 to 250 nM and can be ascribed to the minimal rounds of selection (in this case, two) undertaken with the antigen. It is likely that a scFv fragment with a higher affinity and much lower abundance in the library escaped our detection.

Two of the five scFv fragments isolated from the phage display library showed significant intracellular accumulation where one of them (3C1-A2) was substantially higher than the other (3C1-C4). There could be several reasons for the low intracellular expression of the three remaining scFv fragments. All the scFv constructs were cloned in the same expression vectors (and hence, expression was driven by the same promoter), and the significant DNA sequence identity (68–78%) between the clones suggests that RNA stability and/or transcriptional/translational efficiency was less likely to be the cause(s) of the differential levels of expression observed. Our data imply that protein stability is the defining characteristic that distinguishes between the intracellular expression levels of these scFv fragments. The reducing environment of the cytoplasm (25) precludes the formation of disulfide bonds in scFv fragments (26). A cysteine-less mutant of 3C1-A2 (SS 3C1-A2, all Cys residues were mutated to Ser) was constructed to determine the role of disulfide bonds in the intracellular stability of scFv. Western blotting of lysates isolated from transiently transfected 293T cells demonstrated the accumulation of SS 3C1-A2, albeit to lower levels than the wild-type counterpart (data not shown). This finding illustrates the inherent stability of scFv 3C1-A2, which displays moderate expression levels despite the absence of crucial disulfide bonds. It is possible that Val-Ala replacements for the disulfides would improve on the yield of intracellular soluble protein as suggested by others (21). Intracellular turnover of scFv 3C1-A2 was investigated by pulse-chase experiments and determined to be >12 h (data not shown). The urea denaturation analyses, particularly those conducted in the presence of reducing agent, clearly illustrate the differences in intracellular stability between the scFv fragments. Screening for scFv fragments with moderate to high stability in such reducing and destabilizing environments may be a facile method for obtaining effective intrabodies.

Fig. 9. Urea denaturation hierarchies for the three scFv fragments. (A–C) Denaturation curves for 3C1-D3, 3C1-C4, and 3C1-A2, respectively, illustrating the urea denaturation hierarchy in re-localization efficiencies is mimicked by the level of intracellular accumulation of the individual scFv fragments as depicted by the green FITC staining in the left panels. Similar studies with scFv 3C1-B1 were less informative due to low expression levels of the intrabody.

Intracellular Efficacy in Retargeting the Antigen—The anti-C163S-mCASP3 scFv fragments tagged with SV40 NLS were coexpressed with the antigen in BHK-21 cells to compare their intracellular efficacy in retargeting antigen from the cytoplasm to the nucleus. Representative data from immunofluorescence analyses for 3C1-D3, 3C3-C1, and 3C1-C4 scFv fragments are shown in Fig. 9 (A–C, respectively). A qualitative examination of Figs. 8C and 9 demonstrates that the order of retargeting efficiencies is 3C1-A2 > 3C1-C4 > 3C3-C1 > 3C1-D3 (amount of red staining in the nucleus versus staining in the cytoplasm), as visualized by Texas Red staining in the right panels. This hierarchy in re-localization efficiencies is mimicked by the level of intracellular accumulation of the individual scFv fragments as depicted by the green FITC staining in the left panels. Similar studies with scFv 3C1-B1 were less informative due to low expression levels of the intrabody.
We found that the intracellular stability of an intrabody dictates its efficacy more than its affinity for the antigen. This was apparent when we observed the lower efficiency in redirecting the antigen C163S-mcASP3 by scFv 3C3-C1 (Fig. 9B), despite the stability of the 3C3-C1-mCASP3 complex (66 nM). 3C1-A2, on the other hand, is able to redirect C163S-mcASP3 from the cytoplasm to the nucleus via protein-protein interactions mainly due to its substantial accumulation in the reducing environment of the cell. Similar results have been obtained with intrabodies expressed in mammalian (7) and yeast (27) cells.

The ideal scenario, in which highly stable, specific, and high affinity intrabodies become available, would obviously include the optimization of both the stability and affinity parameters. One way to do this is by constructing a phage display library based on a much more “intracellularly stable” scaffold without compromising too much diversity. Quick screens such as Western blotting of subcellular fractions obtained from transiently transfected mammalian cells and reporter-based assays on whole cell systems can then be employed to determine the intracellular accumulation of isolated moieties. Another method employs a variation of the yeast two-hybrid system to select for functional intrabodies that bind their antigen in vivo (8).

Our ability to isolate a specific and moderate affinity scFv fragment directed against caspase-3 with substantial intracellular availability suggests that this methodology can be employed to generate intrabodies against other intracellular targets. An earlier report from our laboratory (in collaboration

![FIG. 8. Specificity of the intracellular interaction between C163S-mcASP3 and intrabody 3C1-A2 in BHK-21 cells. The left panels depict fluorescence in the FITC channel; the middle panels show bright-field images; and the right panels show the Texas Red channel. A, transiently transfected C163S-mcASP3-FLAG tag fusion in BHK-21 cells. The protein fluoresces in the red channel, whereas the green channel depicts background fluorescence. B, transient cotransfection of 3C1-A2-NLS-Myc tag fusion and non-reactive antigen SHP-1-FLAG tag fusion constructs. The intrabody is visualized in the green channel, whereas SHP-1 is shown in the red channel. C, transient cotransfection of 3C1-A2-NLS-Myc and C163S-mcASP3-FLAG in BHK-21 cells. Again, the intrabody is shown in the green channel, and the antigen is shown in the red channel.](http://www.jbc.org/)

![FIG. 9. Varied efficacies in retargeting the antigen C163S-mcASP3 by three intrabodies, 3C1-D3 (A), 3C3-C1 (B), and 3C1-C4 (C). The left panels depict fluorescence in the FITC channel; the middle panels show bright-field images; and the right panels show the Texas Red channel. C163S-mcASP3-FLAG and the respective intrabody-NLS-Myc constructs were transiently cotransfected into BHK-21 cells. The intrabody is visualized in the green channel, whereas the antigen is shown in the red channel.](http://www.jbc.org/)
Intracellular Stability Determines Efficacy of Intrabody

with IntrAimmune Therapies Inc.) detailed the isolation and efficacy of an anti-caspase-7 intrabody in redirecting the antigen into several subcellular compartments (7). In the current study, we attempted to further elucidate the rules that govern the effectiveness of intrabodies by investigating the stability, affinity, and intracellular accumulation of the largest panel of scFv fragments generated against an antigen thus far, in vitro. We found a correlation between the stability of the individual proteins, as measured by urea denaturation studies, and their respective intracellular concentrations. Recently, our endeavors to test the ability of our leading candidate intrabody (3C1-A2) in redirecting endogenous caspase-3 (and perhaps ablating the activity of the endogenous protein) in mammalian tissue culture have yielded mixed results (data not shown). This result is not unusual due to the existence of other compensatory pathways in apoptosis (involving other downstream caspases such as caspase-6 and caspase-7). Our data reflect other findings that thymocytes isolated from caspase-3−/− mice display similar kinetics of apoptosis as their wild-type counterparts (28). It is also possible that redirecting endogenous caspase-3 to the nucleus might not be sufficient to block apoptosis and cleavage of its substrates.

The current need for functional annotation of putative genes, described by large-scale sequencing efforts, has engendered the development of technologies that will fulfill this requirement. Intrabodies are among several technologies being explored to assist in this task. Our results (including those of others) suggest that the issue of intracellular stability of the scFv fragments in the reducing environment of the cytoplasm (where they are synthesized and folded) is central to their efficacy. Efforts directed at addressing such issues will facilitate the development of intrabodies as an effective tool for functional genomics.

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