Inhibition of Activating Transcription Factor 1- and cAMP-responsive Element-binding Protein-activated Transcription by an Intracellular Single Chain Fv Fragment*

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Activating transcription factor 1 (ATF1) and cAMP-responsive element (CRE)-binding protein (CREB) activate transcription through CREs located in the promoters of cellular and viral genes. We previously described a monoclonal antibody (mAb41.4) that prevents ATF1 binding to DNA and reduces CRE-driven promoter activity in vitro (Orten, D. J., Strawhecker, J. M., Sander-son, S. D., Huang, D., Prytowsky, M. B., and Hinrichs, S. H. (1994) J. Biol. Chem. 269, 32254–32263). A single chain Fv (scFv) fragment from the mAb41.4-expressing hybridoma was generated to provide a means to investigate transcription factor function via intracellular expression of the scFv fragment. The affinity of scFv4 (subgroup: V_L c-III, V_H miscellaneous) for ATF1 was similar to that of the parental mAb and the Fab fragment, but it demonstrated greater inhibitory activity and reacted with CREB. scFv4 disrupted the binding of both ATF1 and CREB in electrophoretic mobility shift assays and reduced expression of CRE-driven expression in vitro. Transient expression of scFv had no effect on the non-CRE-containing adenovirus major late promoter. The proliferating cell nuclear antigen promoter, containing two CREs, was significantly more sensitive to inhibition by scFv than the cytomegalovirus immediate-early promoter, containing five CREs. Cotransfection of either ATF1 or CREB in the presence of scFv restored basal levels of expression. The intracellular expression of scFv provides a unique means to investigate the roles of the transcription factors ATF1 and CREB.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF039853 and AF039854.

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enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility-shift assay; PCNA, proliferating cell nuclear antigen; AdML, adenovirus major late; CMV-IE, cytomegalovirus immediate-early; bZIP, basic leucine zipper.

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of the light and heavy chains connected by a peptide spacer (9–11). When constructed in this manner, a single RNA transcript can be expressed and translated into an active protein that has the potential to interfere with the activity of targeted intracellular proteins. Intracellular scFv fragments have been successfully employed to decrease the expression of Erbb-2 (12, 13); the α subunit of the high affinity human interleukin-2 receptor (14); and proteins of human immunodeficiency virus type 1, including Rev, reverse transcriptase, and envelope protein gp120 (15–18). We therefore cloned scFv4 of mAb4 and present here its characterization and its functional effect on intracellular gene expression.

EXPERIMENTAL PROCEDURES

Preparation of mAb and Fab Fragments—Preparations of mAb4 were affinity-purified on a protein G column and quantitated by absorbance at 280 nm (assuming 1 mg of IgG/ml of IgG = 1.4 A 280) and the Bradford assay (Bio-Rad). Fab fragments were generated as described previously (8). Purified mAb4 was incubated with papain-agarose beads, after which the digested Ab solution was purified away from Fc fragments by protein A column chromatography. Purified Fab4 was dialyzed into phosphate-buffered saline and concentrated by centrifugal ultrafiltration.

Cloning of scFv—The single chain Fv fragment of mAb4 was cloned utilizing a modification of the procedure described by Winter and Milstein (11) and components of the Recombinant Phage Antibody system (Amersham Pharmacia Biotech). Total RNA was isolated from the mAb4.1.4 hybridoma and reverse-primed. The heavy and light variable regions (VH and VL, respectively) were amplified in two separate reactions using degenerate primers (Amersham Pharmacia Biotech) corresponding to the framework regions bracketing the complementarity-determining regions (CDRs) of the VH and VL domains. The resulting polymerase chain reaction products were joined by a linker that encoded a flexible 15-amino acid chain of (Gly-Ser), (Amersham Pharmacia Biotech). The linkage reaction thus generated the scFv cDNA encoding the VH linker-VL domain. The cDNA product was subsequently screened by enzyme-linked immunosorbent assays (ELISAs) using recombinant ATF1 bound to the microtiter wells (7). Positive wells were detected with a horseradish peroxidase-conjugated anti-mIgG Ab (Amersham Pharmacia Biotech). The phages capable of binding ATF1 were used to infect E. coli HB2151 for generation of soluble scFv secreted into the periplasm.

Production and Quantitation of Soluble scFv—Soluble scFv was produced and quantitated as described previously (20). E. coli HB2151 cells that had reached an A 600 of 0.6 were induced with isopropyl-D-thio-galactopyranoside and grown at 25 °C for 4 h. The periplasm was extracted in a high salt lysate buffer, clarified, and dialyzed. Yields averaged 1.5 mg of scFv/filter of culture. scFv quantitation was performed through slot blotting of the periplasmic extract and a peptide standard. The slot blots were stained with an anti-c-myc tag Ab (murine Myc, Jackson ImmunoResearch Laboratories, Inc.). A standard curve (1–100 ng) using c-myc-peptide-1 (Oncogene Scientific Inc.) was generated, and the signal of scFv wells was visually compared for determination of the approximate concentration as well as digitally scanned for densitometric analysis. The cDNA of scFv was normalized for mass (mass of c-myc peptide = mass of scFv/8), the average periplasmic concentration of scFv was observed to be 5 ng/μl. Periplasmic extracts were used to characterize the activity of scFv in ELISAs, Western blots, electrophoretic mobility shift assays (EMSA), and in vitro transcription.

DNA Sequencing and Analysis—Sequencing of the scFv inserts in selected clones was performed through automated sequencing (ABI Advanced Biotechnologies, Inc.). 5′- and 3′-pCANTAB sequencing primers (Amersham Pharmacia Biotech) that anneal to the linker region of the cloned scFv fragment and the poly-cloning site of the pCANTAB vector respectively, were used. The complete sequences of three scFv4 clones (C9, A6, and D6) were determined and compared to confirm the absence of sequence errors and maintain fidelity. The recombinant scFv was translated into amino acid sequence and analyzed using the AbM Version 2.01 software package (Oxford Molecular Ltd., Oxford, United Kingdom) (21–23). Computer analysis identified unusual residues in each Fv fragment and applied the Kabat definitions to define framework regions and CDRs of each Fv fragment. The antibody subgroup and family of each Fv fragment was determined.

ELISA—The ability of scFv to bind ATF1 and CREB was compared with its parental mAb and Fab forms. Competitive ELISAs were performed using the scFv-containing periplasmic extracts and mAb4 or Fab4 as the initial antigen binder. Bound scFv was detected through the use of an anti-c-myc tag Ab and an alkaline phosphatase-conjugated anti-mouse heavy and light chain Ab. Bound mAb4 and Fab4 were detected directly with the anti-mouse heavy and light chain Ab. Competitive ELISAs utilized ATF1- or CREB-coated microtiter wells and full-length ATF1 or CREB and their synthetic subsequences as competitors (7). The competitive inhibitory peptides, peptides C and F, at concentrations of 0.1–10 μM were incubated with solutions of scFv4 periplasmic extract (diluted 1:5 in PBS containing 0.1% Tween 20 and 1% BSA) or Fab4 (dilute phosphatase-buffered saline) before addition to the ELISA plate wells. Negative controls included a competitor peptide unrelated to the sequences of ATF1 or CREB and a non-scFv-containing periplasmic extract generated from E. coli HB2151 cells transformed with the recircularized pCANTAB-5E parental vector (no insert). Results of competitive ELISAs performed with scFv, mAb4, Fab, and negative controls were read on an ELISA plate reader at 405 nm and plotted as inhibitor concentration versus percent inhibition of binding.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed as described previously (7, 8). 32P-Labeled oligonucleotide containing the consensus CRE (5′-AGA GAT TGC CTG CAG TCA GAG ACG TAG-3′) was incubated with either 50 ng of full-length recombinant ATF1 or CREB or one of the deletion mutants: ΔCRRE (ΔZIP region amino acids 254–327) or ΔATP2 (ΔZIP region amino acids 350–505) (Santa Cruz Biotechnology). The binding reactions were carried out in the presence or absence of mAb4, Fab4, scFv4, or the control E. coli. periplasm. Following electrophoresis, the bound and unbound fractions of labeled oligonucleotide were quantitated by autoradiography for 6–12 h using a PhosphorImager (Molecular Dynamics, Inc.). The PhosphorImager data were exported as tagged image file format files to Canvas 5 and used to prepare Fig. 3.

In Vitro Transcription—The effects of scFv on transcription were evaluated as described previously (25) using the HeLa cell nuclear extract in vitro transcription system from Promega. The scFv fragment, antibody, or control periplasm was incubated with nuclear extract containing 5 mM MgCl2 for 30 min before adding ribonucleoside NTPs and transcription complexes used in this assay. One of the templates consisted of the CMV-luc (provided by S. Rhode), which contains CMV-IE promoter sequence −760 to +75 placed upstream of the luciferase reporter gene. Specific RNA transcripts were detected using a 32P-labeled primer complementary to nucleotides +55 to +82 of the luciferase gene and Superscript 1™ RNase H reverse transcriptase (Life Technologies, Inc.). The resultant products of the expected size were quantitated using a PhosphorImager. The values for Fig. 3 were generated by setting the level of product in the presence of the control mAb to 100% and calculating the values for products in the presence of mAb4, Fab4, or scFv according to the level of the control.

DNA Constructs—The pCMV-scFv and pEF-scFv expression vectors were generated by placing the cDNA of scFv clone C9 into the multiple cloning sites of pCMV4 and pEF-1 (provided by Dr. R. Lewis), respectively. pCMV4 incorporates the SV40 origin and the translational enhancer from alpha satellite mosaic virus 4 in addition to the strong CMV-IE promoter (26). pEF-1 is a derivative of the pcDNA3 vector that has the CMV-IE promoter replaced with the promoter region from elongation factor-1α, which contains no CRE sequences (27). BglII and MluI ends were introduced into scFv cDNA by PCR amplification and cloned into the respective sites of pCMV4. The undigested scFv polymerase chain reaction product was ligated into the Invitrogen T/A cloning vector, and the EcoRI-NorI fragment from this ligation was inserted into the appropriate sites of pEF-1.

Transient Cotransfections—Transient cotransfections of HEK293 cells were performed according to established protocols using calcium phosphate co-precipitation.
phate precipitation (28). The transfections were performed three to four times in duplicate 35-mm wells containing one of the reporter constructs (CMV-luc (5 μg), PCNA-luc (10 μg), and AdML-luc (10 μg)) and increasing amounts of the scFv vectors (0, 5, 10, and 20 μg). To control the assays for variations in transfection efficiency, a Rous sarcoma virus-β-galactosidase construct (2 μg) was used as an internal standard. Additionally, in the wells without scFv, a molar equivalent of parent vector was used (without cDNA insert) to maintain an equal number of promoter units in each transfection. The cells were harvested 60–72 h post-transfection, and the reporters were assayed.

Luciferase and β-Galactosidase Assays—Measurement of the reporter activity of firefly luciferase was carried out as described relative to an internal β-galactosidase standard (29). 60–72 h post-transfection, cell extracts were prepared by freeze-thaw lysis in a potassium phosphate buffer. ATP and luciferin were added, and light output was measured with a Luminoskan R5 microplate luminometer (Lab Systems/Denley, Franklin, MA). To measure β-galactosidase, o-nitrophenyl-β-D-galactopyranoside was added, and the absorbance at 405 nm was read on an ELISA plate reader. The luciferase value of each well was normalized to the internal β-galactosidase reporter. Results of three to four experiments were then averaged to generate the data depicted in Fig. 4.

RESULTS

Sequence of scFv4—Our goal of investigating the cellular function of ATF1 and CREB prompted us to clone the scFv fragment of mAb4 from its parental hybridoma cell line. However, the commonly used myeloma cell line fusion partner, P3NS1/1-Ag4-1 (NS1), constitutively secretes a κ light chain (30) that can interfere with the cloning of scFv fragments from these hybridomas. Confirmation that the correct Fv fragment was cloned was obtained by automated sequencing of the scFv fragment. The amino acid sequence of the recombinant Fv fragment was deduced from the nucleotide sequence of cDNA and compared with the previously obtained N-terminal sequence of the parental monoclonal antibody light chain. The N-terminal 55 amino acids of the V\(_λ\) domain of mAb4, extending from framework region 1 through CDR2, were conserved in scFv, confirming that scFv4 did not contain the NS1 κ chain.

Comparisons of the V\(_\text{H}\) and V\(_\lambda\) sequences were made with murine sequences in the Kabat data base of molecules of immunologic interest. The scFv V\(_\lambda\) domain was assigned to the κ-IX family based on nucleotide sequence and placed in subgroup III based on the amino acid sequence of framework region 1. The two sequences in the data base most similar to the scFv V\(_\lambda\) sequence were 48.2.1 (KABAT ID 006048) (31) and PCT043(NZB) (KABAT ID 006046) (32), each with 95% homology. Five unusual residues were present in the V\(_\lambda\) sequence at L3, L48, L50, L63, and L93. Comparison of the V\(_\lambda\) sequence to data base sequences placed it in subgroup IIIH, with six differences from the family definition, and assigned it to a miscellaneous family, indicating that no reference data set correlated with our sequence. The two most homologous sequences were MOPC21 (KABAT ID 003106) (33) and X63.2ARI-16 (KABAT ID 003103) (34), which had 71% homology to our V\(_\lambda\) sequence. Comparison of the scFv V\(_\text{H}\) sequence to MOPC21 and X63.2ARI-16 showed differences prior to H82 at H2, H4, H6, H7, H28, and H71. Up to this point, the sequence correlates with germ-line sequences, but the subsequent 13 residues are significantly different from other mouse sequences. Examination of the nucleotide sequence demonstrated a probable frameshift mutation that accounts for the differences observed between the scFv and germ-line sequences. Shifting the reading frame by one nucleotide results in an open reading frame encoding NYPHYAMDYWGKG that correlates with the framework region H4 sequences of MOPC21 and X63.2ARI-16 at H110–H113. The residues between H82 and H101 (CDR-H3) are absent from the V\(_\text{H}\) chain, and this striking feature is an apparent deletion of CDR-H3 in our scFv fragment.

**scFv4 Maintains the Immunoactivity of mAb4 in Competitive ELISA**—Cloned scFv fragments often maintain the activity of the parental antibody. However, a number of factors may influence the binding characteristics of the scFv fragment, including structural perturbations due to the absence of the constant regions, the linkage order of the variable domains (5’-\(\text{VL-\(\text{VH}\)}\)-3’ versus 5’-\(\text{VH-\(\text{VL}\)}\)-3’), and the composition and length of the linker (35, 36). The initial binding characteristics of scFv4 as compared with mAb4 and Fab4 were evaluated through competitive ELISAs using scFv4, mAb4, Fab4, and a control periplasmic extract (Mock Fv). Competition with recombinant ATF1 bound to microtiter wells was performed with increasing concentrations of the peptides (x axis) as described previously (7, 8). Results are plotted as percent inhibition of binding (y axis).

**Fig. 1. Comparative organization of ATF1 and CREB with epitope of mAb4 and related peptide sequences used in competitive ELISA.** A, functional components of ATF1 and CREB (1) demonstrating the location of the antibody epitope between the transcriptional activation domains and the DNA-binding domains of ATF1 and CREB. NTR, amino-terminal region; Q, glutamine-rich regions; α, α-helical regions; β, β-sheet regions; PκA/PκC, sites for phosphorylation by protein kinases A and C, respectively. B, competitive ATF1 ELISA analysis of scFv4, mAb4, Fab4, and a control periplasmic extract (Mock Fv). Competition with recombinant ATF1 bound to microtiter wells was performed with increasing concentrations of the peptides (x axis) as described previously (7, 8). Results are plotted as percent inhibition of binding (y axis).
CREB, or periplasmic extract, in the presence of recombinant ATF1, CREB, controls included isotype-matched mAb and Fab, as well as a control divergent from ATF1 and CREB in the region of the epitope. Concentration present in full-length CREB.

ATF2 binding to the CRE.

plex formation to 5% of controls (Fig. 2).

scriptional activity of the CMV-IE promoter (8). A clear transcription results showing partial inhibition of the CMV-IE promoter constructs used in the in vitro transcription assays. The CMV-IE promoter contains five CREs, and the PCNA promoter contains two CREs (shown as shaded boxes). The AdML promoter is a minimal promoter containing a TATA box and two Sp1/MAZ sites and does not contain a CRE. B, comparison of the effect on transcription formation in in vitro transcription assays using HeLa cell nuclear extracts with 10 μg of mAb4, 7 μg of Fab4, and 1 μg of scFv4 (a one-third valence equivalent to the mAb or Fab fragment used). Controls included an isotype-matched mAb (10 μg) and a mock Fv fragment (used at an equal volume to scFv4) to control for possible effects of periplasmic extract constituents.

scFv4 Prevents Transcription from CRE-driven Promoters in Vitro—The binding of DNA by transcription factors and the process of transcriptional activation are related but separate functions. Since the scFv fragment prevents binding of ATF1 to DNA, the reduction of transcriptional activation was the anticipated secondary effect. In vitro transcription experiments were therefore important to determine whether the scFv inhibition of ATF1 and CREB binding to DNA resulted in reduced levels of transcription. Fig. 3A shows a comparison of the promoter controls used in the in vitro assays. Our interest was to determine whether the apparent reactivity of scFv4 for ATF1 and CREB in EMSA would correlate with inhibitory activity using a promoters such as those of PCNA and CMV-IE. As a control for nonspecific interference of transcription, the AdML promoter was chosen. The AdML promoter (Fig. 3A) is a minimal promoter containing a TATA box and a pair of MAZ and Sp1 elements (38–40). There are no CREs or CRE-like sequences present, allowing for demonstration of specificity of the Recombinant CRE and scFv4. scFv4 was found to inhibit transcription from the AdML promoter (Fig. 3B), comparable to the level of inhibition achieved previously with the mAb and Fab fragment (7, 8). Our previous experiments demonstrated that Fab4 reduced the production of product expressed from the CMV-IE promoter by 93% (Fig. 3B), comparable to the level of inhibition achieved previously with the mAb and Fab fragment (7, 8). We observed that incremental increases of scFv added to transcription reactions progressively decreased the intensity of transcript product to approximately two-thirds of that with Fab4. No effect was observed on the production of transcription product from the AdML promoter in the presence of Fab4, mAb4, or scFv4. The control periplasmic extract did not alter expression of the transcription products from these promoters, nor did control antibodies.

Intracellular Expression of scFv Interferes with CRE-driven Transcription—HeLa cells were chosen to determine the intracellular effects of scFv as in vitro transcription assays had been performed using HeLa cell nuclear extracts. ATF1 has been shown to be expressed at significantly higher levels than CREB in HeLa cells (4); therefore, we expected to observe significant expression from the CRE-driven luciferase reporters. The three...
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separate luciferase reporters (CMV-luc, PCNA-luc, and AdML-luc) used in the in vitro transcription assays were cotransfected with either scFv expression vectors or control vectors without a cDNA insert. To control the experiments for variations in transfection efficiency, a Rous sarcoma virus-β-galactosidase construct was used. By assaying the β-galactosidase activity and normalizing for transfection efficiency, the cellular transcriptional activity was compared between experiments and the different constructs. A base line for each reporter was thereby established. The strong CMV-IE-luc reporter produced ~50 relative light units/μl of lysate, whereas the weaker PCNA-luc and AdML-luc reporters generated 0.3 and 0.02 relative light units/μl, respectively.

Two different scFv-expressing constructs, pEF-scFv and pCMV-scFv, were utilized to determine the intracellular effect of scFv on CRE-driven transcription. The pEF-1 construct contains the promoter of the elongation factor-1 gene that is expressed continuously at a basal level. The pCMV construct contains the CMV-IE promoter and was expected to express high levels of scFv4 before a potential negative feedback effect occurred. In transient cotransfection assays, the pCMV-scFv construct was found to decrease the expression of CMV-luc by 2–3-fold more than the pEF-scFv construct; therefore, the pCMV-scFv construct was chosen to perform further comparisons.

The effect of scFv activity on each promoter was determined using four different levels of pCMV-scFv (0, 5, 10, and 20 μg). Each level of pCMV-scFv was balanced with an amount of parental pCMV4 to obtain a final molar concentration equivalent to 20 μg of pCMV4-derived DNA/well. Progressively greater amounts of scFv added to the CMV-luc system (Fig. 4, upper left panel) resulted in corresponding decreases in luciferase expression: 18% at the 10-μg level and 25% at the 20-μg level. The PCNA promoter was more sensitive to the inhibitory effects of scFv than the CMV-IE promoter (Fig. 4, lower panel). A 30% decrease was observed in PCNA-luc expression in the presence of 5 μg of pCMV-scFv, and transfection of the scFv vector reduced the expression of PCNA-luc in a dose-dependent manner. 10 μg of scFv vector reduced luciferase expression by 44%, and 20 μg of scFv vector decreased luciferase expression by 56% of control values. The AdML-luc construct that contains no CREs was not affected by the presence of pCMV-scFv (Fig. 4, upper right panel), demonstrating that the activity of scFv was specific for CRE-driven transcription. Further demonstration of the specific activity of scFv was obtained by cotransfection of either ATF1- or CREB-expressing constructs into the HeLa cells. The overexpression of either ATF1 or CREB was capable of reversing the inhibition by scFv of the luciferase reporters (Fig. 4, lower panel).

**FIG. 4.** Expression of scFv decreases CRE-driven transcription in HeLa cells. Transient cotransfection experiments in HeLa cells were performed using 5 μg of CMV-luc (upper left panel) and 10 μg of PCNA-luc (lower panel) or AdML-luc (upper right panel) as reporters. Relative light units (RLU) are depicted on the y axis, with results of reporter alone set to 1.0 for comparison. Bars represent averages of three to four experiments, with means (±) and standard errors (σ) shown below. Experiments used increasing amounts of pCMV-scFv balanced with parental pCMV4 to achieve a constant number of CRE sequences in each reaction. Differences in transfection efficiency were controlled through cotransfection of a Rous sarcoma virus-β-galactosidase construct.

**DISCUSSION**

Although studied extensively, the cellular roles of ATF1 and CREB have not been fully determined. CREB is an important mediator of cAMP-activated transcription, whereas ATF1 activates transcription in some cell lines (41) and represses transcription in others (3). To elucidate the roles of transcription factors, investigators have attempted to knock out the activity of the transcription factors. In studies with ATF1 and CREB, activity has been reduced in vitro with an ATF1-sequestering CRE oligonucleotide (42) and in situ through the generation and study of transgenic and mutant mice (6, 43, 44). Other investigators have used intracellularly expressed scFv fragments as modified gene knockout systems (12, 13). The recent success in the application of these modified knockout systems led us to evaluate the inhibitory activity of the scFv fragment derived from mAb4.

The ATF1 binding activity of mAb4 was maintained by scFv in ELISA (displaying a similar affinity for ATF1). In EMSA, scFv inhibited the formation of ATF1-CRE complexes, as had occurred with the mAb, and also inhibited the formation of CREB-CRE complexes, similar to the effect of the Fab fragment. Previous studies in our laboratory demonstrated that both mAb4 and Fab4 inhibited transcript production from the PCNA promoter (7, 8). The inhibition of ATF1-CRE and CREB-CRE complexes in EMSA correlated with reduction of expression from the CRE-containing promoters in in vitro transcription assays. The cell transfection experiments confirmed the inhibitory effect of scFv on CRE-containing promoters. Therefore, the EMSA and in vitro transcription studies were predictive of the effect of scFv in cells, but the specific level of inhibition was different in each assay.

The mAb interfered solely with ATF1, whereas the Fab fragment possessed additional reactivity for CREB as observed by EMSA and in vivo transcription from the CMV-IE promoter. The scFv fragment maintained reactivity for CREB, but the affinity was lower for CREB than for ATF1. Although the scFv fragment has an apparent lower affinity for CREB than for ATF1, it has greater inhibitory activity than the Fab fragment or mAb in vitro and maintains this activity in cells. The apparent difference in the relative activities of the scFv construct and its parental mAb for the two transcription factors could derive, in principle, from several factors that are still to be determined. First, structural constraints in the antigen-binding site may be imposed by the covalent linkage of the VL and VH domains via the 15-residue linker (Gly3Ser)3 or by the order of the two domains (the VL C terminus linked to the VH N terminus versus the VH C terminus linked to the VL N
transmitter as in this study). Although scFv constructs generally retain the activity of the parental antibodies, the length and composition of the linker (35, 45), as well as the assembly order of the V\textsubscript{L} and V\textsubscript{H} domains, have been reported to influence the antigen binding affinity and avidity in certain cases (36, 45). Second, an increased ability of scFv to diffuse into small spaces, due to the smaller size of the former molecule (27 kDa versus 150 kDa for the mAb), may increase the accessibility of the antigenic epitope within the transcription factor-DNA complex.

The tertiary structure of CREB may be such that certain domains are accessible by the small scFv fragment, but not by the large intact antibody. Third, aggregation reactions are known to occur in certain Fv preparations (46, 47), which may alter the avidity of the binding for the two transcription factors. Significant intermolecular noncovalent interactions between the variable domains present in different scFv molecules could potentially occur at the scFv concentrations employed in our experiments (0.3–3 μM), depending on the affinity constant for noncovalent V\textsubscript{L}-V\textsubscript{H} complexations. Fourth, the presence of unusual residues in our scFv fragment may indicate that nonsomatic mutations are responsible for the additional activity. The scFv fragment is notable for the lack of a CDR3 in its VH domain. The CDR3 deletion in our scFv fragment most likely arose from a somatic event since nucleotide sequencing of multiple isolated clones indicated the occurrence of this deletion, and examination of the sequence data revealed a frameshift occurring at this point in the V\textsubscript{H} domain. In consideration of the above issues, we propose that scFv binds to ATF1 or CREB and limits transcription factor interaction with the CRE. Interference with the binding of ATF1 or CREB to the CRE is believed to prevent the recruitment of transcriptional machinery and subsequent gene expression. Although the reactivity of scFv for both ATF1 and CREB is a useful property, further affinity maturation toward either transcription factor may allow specific inhibition of biological activity.

Multiple approaches were taken to demonstrate that the activity of scFv was specific for the CRE-binding proteins, ATF1 and CREB. The adenovirus major late promoter was chosen because it contains only a TATA box and a pair of MAZ and Sp1 elements (38–40). No effect on the PCNA promoter compared with the CMV-IE promoter, with 56% inhibition of scFv shown (24). The CMV promoter we used contains region 1 through 143 of the promoter and 760 through 182 to 276 of the promoter and 201 through 423 of the promoter and 3137 through 3141 of the promoter and 349 through 353 of the promoter and 272 through 11968 to 11974 of the promoter and 272 through 11968 to 11974 of the promoter and 272 through 11968 to 11974 of the promoter and 272 through 11968 to 11974 of the promoter. In our transfection studies, scFv showed a greater inhibitory effect on the PCNA promoter than the CMV-IE promoter, with 56 and 25% decreases in the promoter activities, respectively. The PCNA construct encompasses 182 to +143 of the promoter and contains single Ap2, Oct, and Sp1 elements (38–40). No CRE or CRE-like sequences are present in the AdML promoter. Additionally, adding back ATF1 or CREB to the intracellular scFv system reversed the effects of scFv upon the PCNA promoter.

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