Loss of Heterochromatin Protein 1 (HP1) chromodomain function in mammalian cells by intracellular antibodies causes cell death

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Summary

The chromodomain (CD) is a highly conserved motif present in a variety of animal and plant proteins, and its probable role is to assemble a variety of macromolecular complexes in chromatin. The importance of the CD to the survival of mammalian cells has been tested. Accordingly, we have ablated CD function using two single-chain intracellular Fv (scFv) fragments directed against non-overlapping epitopes within the HP1 CD motif. The scFv fragments can recognize both CD motifs of HP1 and Polycomb (Pc) in vitro and, when expressed intracellularly, interact with and dislodge the HP1 protein(s) from their heterochromatin localization in vivo. Mouse and human fibroblasts expressing anti-chromodomain scFv fragments show a cell-lethal phenotype and an apoptotic morphology becomes apparent soon after transfection. The mechanism of cell death appears to be p53 independent, and the cells are only partly rescued by incubation with the wide spectrum caspase inhibitor Z-VAD fmk. We conclude that expression of anti-chromodomain intracellular antibodies is sufficient to trigger a p53-independent apoptotic pathway that is only partly dependent on the known Z-VAD-inhibitable caspases, suggesting that CD function is essential for cell survival.

Key words: Intracellular antibodies, scFv fragments, Chromodomain, HP1 proteins, Heterochromatin, Apoptosis

Introduction

Recent insight into the molecular nature of heterochromatin has come from the isolation of genetic modifiers that can either suppress or enhance position effect variegation (PEV) in *Drosophila* (Tartof et al., 1989; Henikoff, 1990; Singh and James, 1995). Suppressors of PEV are thought to regulate the formation of multi-component complexes through being either structural components of heterochromatin or enzymes that modify these components (Reuter and Spierer, 1992). The first structural component to be identified was heterochromatin protein 1 (HP1) (James and Elgin, 1986). HP1 was shown to be a dominant dosage-dependent modifier of PEV (Eissenberg et al., 1990). The null mutant is lethal, most probably because of defects in segregation of chromosomes in addition to ectopic telomere-telomere fusions (Kellum and Alberts, 1995; Fanti et al., 1999). HP1 also shares a highly conserved motif, called the chromodomain (chromatin organization modifier domain; CD) with another *Drosophila* protein, Polycomb (Pc), which is a repressor of the homeotic genes (Paro and Hogness, 1991; Singh et al., 1991). The identification of the chromodomain in Pc suggested that repression of the homeotic genes had similarities to heterochromatin-induced silencing (Gaunt and Singh, 1990; Paro, 1990), and there are now several lines of evidence to support this (Pirrotta, 1995; Cavalli and Paro, 1998).

The chromodomain motif is highly conserved, which allows the isolation of HP1 and Pc-like proteins from mammals (Singh et al., 1991). There are three HP1 proteins in mammals. In mouse, they are designated HP1α, M31 (HP1β) and M32 (HP1γ), and in man HP1α, HP1β and HP1γ. HP1 proteins have a similar structural organization (Jones et al., 2000), are small (around 200 amino acids) and possess a CD at the N-terminus, which is immediately preceded by a stretch of negatively charged amino acids (usually glutamic acid). The CD consists of anti-parallel, three-stranded β-sheets packed against an α-helix (Ball et al., 1997). Towards the C-terminus, HP1 proteins contain a related sequence, known as the chromo shadow domain (CSD) (Aasland and Stewart, 1995). At the level of 3D structure, CD and CSD show remarkable similarity to the histone-like archeobacterial proteins Sac7d and Sso7d but lack the surface charge that is necessary for DNA binding (Ball et al., 1997; Brasher et al., 2000).

In mammals, the functions of HP1 proteins are wide ranging (Jones et al., 2000). HP1β is a dosage-dependent modifier of a variegating position-effect (Festenstein et al., 1999), like *Drosophila* HP1. This ability to silence gene activity has been utilized by mammalian transcriptional repressors: there is good evidence that HP1 proteins are recruited by several transcriptional repressors, including the KRAB-ZFPs, which are the largest family of repressors in mammals (Ryan et al., 1999). The mechanism by which HP1 proteins repress gene activity has become clearer from a known interaction with the histone methyl-transferase Suv(3)9h1 (Aagaard et al., 1999; Melcher et al., 2000). It is thought that methylation of H3 histone at lysine 9 forms a substrate for binding of HP1 proteins and repression (Rea et al., 2000; Lachner et al., 2001;

Bannister et al., 2001). This role for HP1 proteins in silencing gene activity is likely to be conserved from yeast to man (Wang et al., 2000).

A role in the recruitment of nuclear envelope (NE) precursors at the end of mitosis has also been shown (Kourmouli et al., 2000). HP1β and HP1γ accumulate at the polar surfaces of both human and murine anaphase chromosomes. This polar accumulation closely resembles the cap-like structure that develops at the early stages of NE re-assembly (Georgatos and Theodoropoulos, 1999). Consistent with a role in nuclear envelope re-assembly, an N-terminal fragment of HP1β, which contains a NE-binding site, acts as a dominant negative and abolishes targeting of membrane proteins to the surfaces of chromosomes (Kourmouli et al., 2000). Thus, the presence of HP1 proteins at the polar surfaces of anaphase chromosomes seems to provide a 'platform' that is used by NE precursors for the assembly of a new nucleus. An additional mitotic role for HP1 proteins is also suggested from the study of INCENPs, where HP1β is required for targeting of this protein to the mitotic spindle (Ainsztein et al., 1998).

Finally, cytogenetic studies have indicated two roles for HP1 proteins during mammalian spermatogenesis. The first is in sex chromosome inactivation (Motzkus et al., 1999; Hoyer-Fender et al., 2000). The second concerns a role in chromosome cohesion, which is required for appropriate segregation of the sex chromosomes during male meiosis (Turner et al., 2001).

In order to determine the importance of HP1 chromodomain proteins for cell survival, we have used the intracellular antibody approach (for reviews, see Biocca and Cattaneo, 1995; Cattaneo and Biocca, 1997). Intracellular antibodies, in particular single-chain Fv (scFv) fragments, have been successfully expressed inside cells to ablate the function of oncogene products (Biocca et al., 1993), HIV viral proteins (Mhashilkar et al., 1995; Levy-Mintz et al., 1996) and demonstrated to induce viral resistance in plants (Tavladoraki et al., 1993). The functional basis of intracellular antibodies is closely linked to their ability to interact with their target antigens in vivo. This interaction allows either a direct neutralizing effect or the dislodgment of the antigen from its normal intracellular location which, by this mechanism, inactivates its function (Lener et al., 2000; Cardinale et al., 2001).

In this paper we report the cloning and intracellular expression of two scFv fragments directed against non-overlapping epitopes of the HP1 CD motif. Different constructs have been designed to express the anti-CD scFv fragments in the nucleus and in the cytoplasm of mammalian cells. The intracellularly expressed scFv fragments are shown to interact with and dislodge the HP1 protein(s) from their heterochromatin localization in vivo. In vivo interaction and inhibition of the endogenous HP1 CD proteins leads to a cell-lethal phenotype. Cells undergo an irreversible p53-independent cell death program, suggesting that there is an absolute requirement for HP1 chromodomain function in mammalian cells.

Materials and Methods

Engineering of the 372 and 373 scFv fragments.

Hybridoma V regions have been cloned using a phage display system (Barbas et al., 1991; Krebber et al., 1997). Briefly, mRNA was prepared from the MAC 372 and MAC 373 hybridoma cells, as described previously (Chomczynski and Sacchi, 1987). *VH* and *VL* genes were amplified from the cDNA derived from both hybridomas using selected oligos (Orlandi et al., 1989). For the amplification of the light chains, a mixture of five 3′-primers (VKFOR mix) and a mixture of eighteen 5′-primers (VKBACK mix) were used. For the amplification of the heavy chains, mixtures of five 3′ primers (VHFOR mix) and twenty 5′ primers (VHBACK mix) were used. The *VH* and *VL* repertoires were separately cloned into the pDAN vector for growth of phage(mid) particles (Sblattero and Bradbury, 2000).

Expression of scFvs at the surface of the filamentous phage M13 and phage ELISA

pDAN-transformed colonies of *E. coli* DH5αF′ bacteria were infected with the helper phage M13K07 (Amersham Pharmacia) to a final concentration of 10^{10} pfu/ml for 30 minutes at 37°C, and, after centrifugation, the bacteria were grown overnight at 30°C in fresh LB, containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. Supernatants, containing scFv phages, were analyzed for binding to coated HP1β-GST in phage-enzyme-linked immunosorbent assay (ELISA) (Roovers et al., 1998). Positive clones were further analyzed by fingerprinting and sequencing.

scFv purification

Anti-lysozyme scFv D1.3 (Hawkins et al., 1993) (kindly provided by G. Winter) and selected anti-chromodomain scFv fragments were expressed in *E. coli* HB2151 non-suppressor strain. 1 l of transformed bacteria was induced by 1 mM isopropylthiogalactoside for 5 hours at 37°C, and the extracts were obtained by lysis on ice for 30 minutes in 20 ml buffer (8 M urea, 0,1 M NaH_2PO_4; 0,01 M Tris-Cl, pH 8 containing 1 mg/ml lysozyme) and sonication. The scFvs were purified by affinity chromatography using Ni-NTA agarose (Qiagen) and analyzed by SDS/PAGE followed by western blotting using the mouse anti-tag monoclonal antibody SV5 (Invitrogen).

ELISA assays

Purification of recombinant HP1α, HP1β, HP1γ, mPc1 (M33) and CD expressed as fusion proteins with GST was performed as described previously (Smith and Johnson, 1988). The ELISA assay was carried out by coating the plate with a solution of 10 μg/ml GST fusion proteins in PBS and a solution of 3mg/ml of lysozyme (Sigma). After saturation with 2% w/v BSA/PBS solution, 10 μg/ml scFv fragment solution (diluted in PBST 2% BSA) was added. Detection was performed using mouse anti-SV5-tag monoclonal antibodies (Invitrogen) and goat anti-mouse linked horseradish peroxidase (Amersham Pharmacia)

The competition ELISA was carried out by coating with 50 μl of 10 μg/ml scFv fragment solution. After saturation, 100 μl of 5 μg/ml HP1β-GST protein, which had been previously incubated for 30 minutes at room temperature with or without the purified anti-CD scFv fragments or the non-relevant anti-lysozyme D1.3 at a 1:10 molar ratio, was added to the coated wells. The HP1β-GST bound protein was determined by further incubation with goat anti-GST antibodies (Amersham-Pharmacia) and rabbit anti-goat linked horseradish peroxidase (Pierce).

DNA constructs

For the expression in mammalian cells, all scFv fragments employed in this work were subcloned into the *Nco*I/*Not*I sites of pscFvexpress-cyt and pscFvexpress-nuc vectors (Persic et al., 1997). For PCR amplification of anti-CD scFv fragments from the bacterial pDAN-scFv vector, the following degenerate primers were designed: 5′-

GCAGCAAGCGGCGCCCATGGCC and 3′-TTTGGGATTGCGGC-CGCGCTAGC. The pscFvexp-cyt-163R4 (anti β-gal), which was derived from pPM163-R4 (kindly provided by P. Martineau), was subcloned as described previously (Lener et al., 2000).

Cell lines, transfection, immunoprecipitation and western blotting

Simian COS fibroblasts, murine NIH-3T3 fibroblasts, human embryonal kidney HEK-293 (kindly provided by R. Testi, University of Tor Vergata, Rome) and human osteosarcoma Saos-2 p53 null cells (kindly provided by G. Melino, University of Tor Vergata, Rome) were grown in DMEM medium supplemented with 10% fetal bovine serum.

COS and NIH-3T3 cells were transiently transfected as described previously (Cardinale et al., 2001). HEK-293 cells were transfected with Superfect (Qiagen) and Saos-2 cells with lypofectamine 2000 reagent (Life Technologies) following the manufacturer's instructions, with a DNA/transfectant reagent ratio (w/v) of 1:5 in both cases. Z-VAD fmk (N-benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone) (Vinci-Biochem) was used at a final concentration of 30 μM and added either before or after transfection; it did not affect the rescue of apoptotic cells.

Cells were harvested and analyzed 48 hours after transfection. Lysis, extraction of cellular proteins from transfected cells, immunoprecipitation and western blotting were performed as previously reported (Cardinale et al., 2001). An immunoblot of scFv fragments was performed using the anti-myc IgG 9E10 antibodies and immunoprecipitation by using the same affinity-purified antibody linked to Protein-A Sepharose. Immunoblotting to detect HP1β was carried out with rat anti-HP1β antibody MAC 353 (Wreggett et al., 1994). The following secondary antibodies were used: sheep anti-mouse IgG horseradish peroxidase (Amersham-Pharmacia) and goat anti-rat IgG horseradish peroxidase (Pierce).

Immunofluorescence

Immunofluorescence was carried out as described previously (Lener et al., 2000). Incubations with affinity-purified mouse anti-myc IgG 9E10, rat anti-HP1β IgG (MAC 353), rat anti-HP1γ IgM (MAC 385), rat anti-M33 IgM (MAC 402) and rabbit anti-HP1α IgG (M235) were performed at room temperature for 1 hour. Fluorescein-isothiocyanate-conjugated (FITC), goat anti-mouse IgG (Pierce), Texas Red anti-mouse IgG (Calbiochem), FITC-conjugated goat anti-rat IgG (Sigma), tetramethyl-rhodamine isothiocyanate (TRITC) rabbit anti-rat IgG (Sigma), biotin-conjugated goat anti-rat IgM (Pierce), rhodamine-conjugated goat anti-rabbit IgG (Sigma) and Texas-Red streptavidin (Amersham-Pharmacia) were used for detection. The dye Hoechst 33258 was used at a 1 μg/ml concentration. Samples were examined with a Leica fluorescence microscope and CCD camera, equipped with a 100× oil immersion lens.

In situ identification of apoptotic cells

Apoptotic cells were visualized by staining with the blue fluorescent dye Hoechst 33342 (Sigma) and a phosphatidylserine assay. Hoechst 33342 was used at different concentrations, depending on the cell line: 0.3 μg/ml for COS, 0.25 μg/ml for NIH-3T3, 0.05 μg/ml for HEK-293 and 0.125 μg/ml for Saos2 cells. Phosphatidylserine assay was performed using Annexin-V-FLUOS staining kit (Boeringher-Roche) following the manufacturer's instructions.

Annexin-V- and Hoechst-33342-positive cells were counted from non transfected cells and cells transfected with different scFv fragments. The results shown in Figs 6 and 7 are the average from three different experiments for each cell line used. At least 50 positively transfected cells for each plasmid were counted and checked for their positive reaction to phosphatidylserine (Annexin V) and Hoechst 33342.

Results

Phage-display cloning of anti-chromodomain scFv antibodies

A common strategy for cloning specific V regions from hybridoma cell lines involves PCR amplification of the V regions using primers in or around the V domains, their assembly into a single chain Fv format and expression of scFv fragments as secreted proteins in bacteria. Finally, they were tested for specificity using antigen-binding assays. This is the method of choice when starting with a hybridoma cell line that contains single functional heavy and light chains, which encode the binding specificity of the antibody. However, in the two hybridomas used in this study we found that a functional light chain from the myeloma fusion partner was also present, so we decided to use the phage display technique as an alternative strategy. Accordingly, the V regions of rat monoclonal antibodies MAC 372 and MAC 373 were PCR amplified from hybridoma mRNA and cloned into pDAN, which displays the expressed scFv on the surface of the

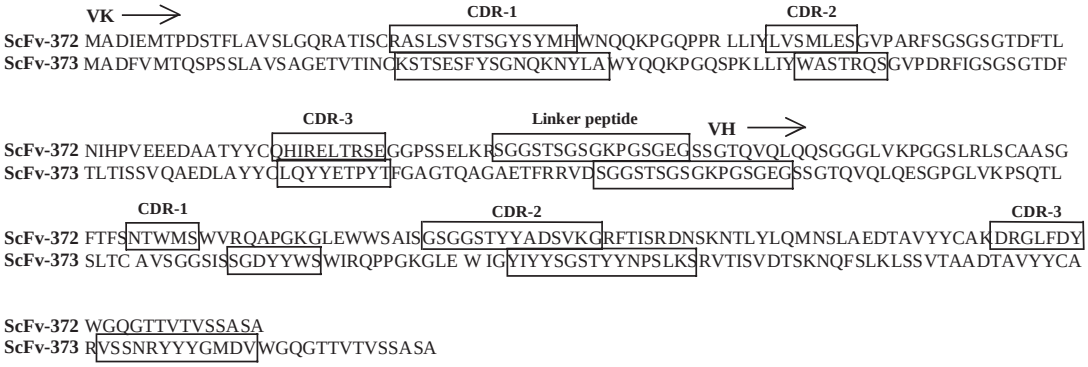

Fig. 1. The amino-acid sequences of 372 and 373 scFv fragments. The deduced amino-acid sequences of the VK and VH variable regions of 372 and 373 antibodies assembled in a VK-VH single-chain format are shown. Complementarity determining regions (CDRs) and the linker peptides are boxed.

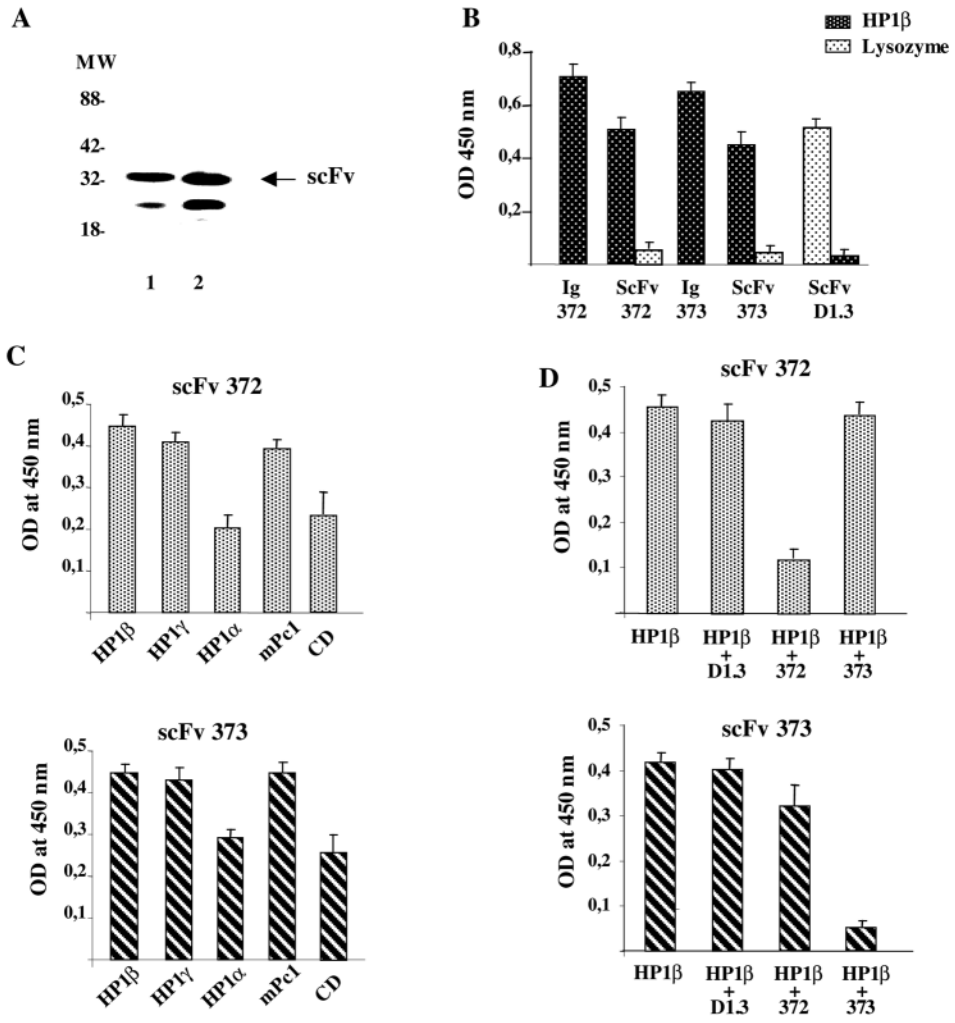

Fig. 2. Expression of the 372 and 373 scFvs in *E. coli*. (A) Western blot analysis of the purified His6-tagged scFv 372 (lane 1) and 373 (lane 2) obtained by affinity chromatography. The arrow points to the scFv protein. The migration of molecular weight markers (in kDa) is shown on the left. (B) An ELISA to measure and compare the binding activity of purified scFv fragments and the parental Ig. A solution of 10 μg/ml of HP1β-GST protein (dark columns) and 3 mg/ml of lysozyme (light columns) was used for coating. As a negative control, a purified anti-lysozyme D1.3 scFv fragment was used. Values obtained by coating the wells with GST alone have been subtracted. (C) An ELISA to determine the fine specificity of the scFv fragments against recombinant chromodomain-containing proteins HP1β, HP1γ, HP1α, mPc1 and the chromodomain (CD) peptide, all fused to GST. Values obtained by coating the wells with GST alone have been subtracted. (D) An ELISA to test competition of anti-chromodomain scFv fragments against the same epitope. Miniwells were coated with soluble purified 372 (upper panel) or the 373 scFv fragment (lower panel). HP1β-GST protein, which had been previously incubated for 30 minutes at room temperature with or without the purified anti-CD scFv fragments or the non relevant anti-lysozyme D1.3 scFv at a 1:10 molar ratio, was added to the coated wells. The HP1β-GST bound protein was determined by further incubation with anti-GST antibodies and anti-goat-peroxidase.

filamentous M13 phage by fusion with the gene III minor coat protein (g3p) (Barbas et al., 1991; Sblattero and Bradbury, 2000). MAC 372 and MAC 373 were raised against the chromodomain of M31(HP1β) protein and shown to be specific by ELISA (data not shown) (Fig. 2B). A restricted phage display library was made for each hybridoma. Phage-ELISA was then used to screen phages for recombinant HP1β-GST protein and two control antigens, lysozyme and GST. Phages specific for HP1β were fingerprinted and sequenced. Fig. 1 shows the amino-acid sequences of MAC 372 and 373 anti-chromodomain scFv fragments as deduced from the nucleotide sequences.

Recombinant anti-HP1β scFv fragments were expressed in bacteria, purified by nickel chelation chromatography and analyzed by western blotting using the anti-tag SV5 monoclonal antibody (see Materials and Methods). As shown in Fig. 2A, a major immunoreactive band of expected size (32 kDa) is present (372scFv, lane 1; 373scFv, lane 2). A less abundant faster migrating band is also present in each of the lanes, which probably represents a degradation product. Coomassie-blue staining of the same gel shows no other contaminating bands, indicating that the preparations are more than 95% pure. The two purified scFv fragments were characterized for binding specificity using ELISA and shown to maintain the same immunogenic properties of the original monoclonal antibodies MAC 372 and MAC 373 (Fig. 2B). Also, as shown in Fig. 2C, the two scFv fragments recognize other CD proteins, namely HP1γ, mPc1 (M33) and, to a lesser extent, HP1α. Binding is likely to be via the chromodomain because scFv antibodies bind to a CD-GST fusion, albeit at a reduced level when compared with the full-length HP1β protein. The reduced binding to the CD-GST fusion is most probably because this fusion aggregates when stored in a purified form. This results in less soluble material for coating of each ELISA well.

The scFv fragments bind non-overlapping epitopes within the CD

Using a competitive ELISA assay we next tested whether the

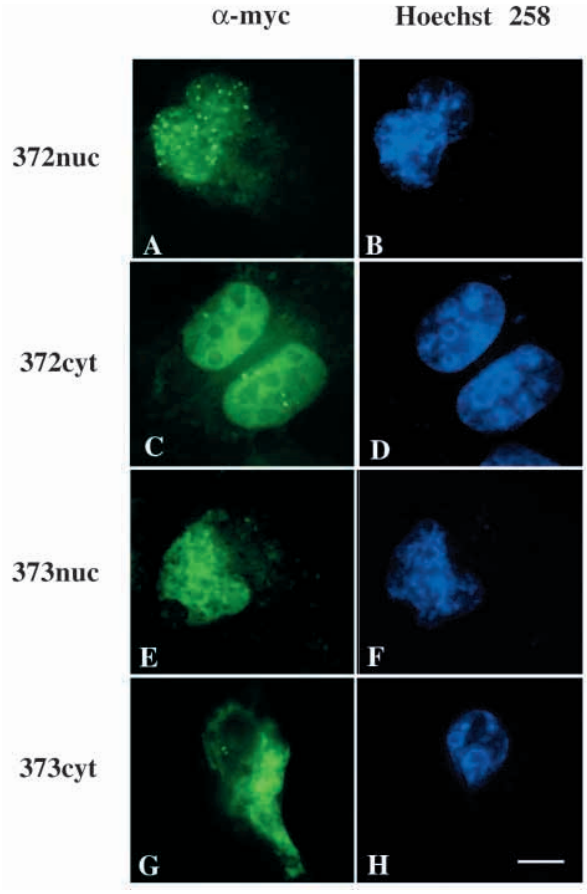

Fig. 3. Expression of anti-CD scFvs in mammalian cells. COS cells were transiently transfected with the 372 scFv fragment targeted to the nucleus (372nuc) (A), 372 targeted to the cytoplasm (372cyt) (C) and with the 373nuc (E) and 373cyt (G). Their nuclear localization was determined using anti-myc antibodies. In the corresponding right panel (B,D,F,H) are shown the same cells counterstained with the dye Hoechst 33258, which highlights the A/T-rich repeat sequences present in the prominent heterochromatic regions. Scale bars, 5 μm.

scFv fragments recognize different epitopes within the CD (Fig. 2D). In this experiment, ELISA plates were coated with each of the purified scFv fragments: 372 in Fig. 2D, upper panel and 373 scFv fragment in Fig. 2D, lower panel. The wells were next incubated with purified HP1β-GST protein, either on its own or after prior incubation with the purified anti-CD scFv fragments or a non-relevant anti-lysozyme D1.3 scFv. The HP1β-GST bound by the scFv coating the wells was detected by further incubation with anti-GST antibodies. As shown (Fig. 2D), when HP1β is preincubated with the same anti-CD scFv fragment used for coating, the amount of HP1β bound is significantly reduced. In contrast, when HP1β is preincubated with a different anti-chromodomain scFv fragment from that coating the wells, or with the anti-lysozyme scFv, there is no inhibition of the binding.

These data demonstrate that the scFv fragments bind non-competitively and therefore each recognizes a unique epitope within the HP1β CD.

Expression of anti-chromodomain scFv fragments in mammalian cells

The 372 and 373 scFv fragments were subcloned into vectors optimized for intracellular expression in mammalian cells (Persic et al., 1997). For nuclear targeting, three nuclear localization signals (NLS) PKKKRKV of the large T antigen of SV40 virus were tagged to the C-terminal of the scFvs, as this signal has been shown to efficiently direct antibody fragments to the nucleus (Biocca et al., 1995).

Immunofluorescence analysis of transiently transfected COS and NIH-3T3 fibroblasts revealed that the intracellular expression of anti-CD scFv fragments cause a cell-lethal phenotype. Most of the transfected cells are round and tend to detach from the dish, indicating that CD function is required for cell survival. This is not a general cytotoxic effect of intracellular antibodies because expression of scFv fragments against non relevant antigen, such as, for example, anti-βgal, did not result in cell sickness (Figs 6 and 7). Notwithstanding this highly toxic effect, some transfected cells retain normal nuclear morphology, and this has allowed us to study the intracellular localization of the expressed scFv fragments. In particular, 372 anti-CD scFv fragments localize to the nucleus regardless of whether the scFv contained a NLS or not (Fig. 3A,C), giving rise to two major types of pattern – a punctate pattern on the background of a more uniform staining (Fig. 3A) and another where there is only uniform staining of the nucleus with few nuclear aggregates (Fig. 3C). The percentage of cells displaying nuclear aggregates varies between 50 and 70 depending the cell line transfected. Nuclear localization was also obtained with the nuclear version of 373 (Fig. 3E), although 373cyt showed only a partial localization to the nucleus, with the bulk of 373cyt remaining in the cytoplasm (Fig. 3G).

The simplest explanation for the observation that cytoplasmic 372 anti-chromodomain scFv can localize to the nucleus (Fig. 3C) is that the interaction between the intracellularly expressed scFvs and the NLS-containing HP1 proteins occurs in the cytosol. The antigen-antibody complex is then transported across the nuclear membrane into the nucleus because of the NLS present in HP1 proteins.

Coimmunoprecipitation of HP1 proteins and anti-chromodomain scFvs

Guided by our observations we next wanted to investigate whether the scFvs do, in fact, interact with HP1 proteins in vivo. In order to do this we performed coimmunoprecipitation experiments. COS cells were transfected with 372-nuc and 373-nuc scFv fragments and analysed after 48 hours. The scFvs were then immunoprecipitated from cell extracts using the anti-myc 9E10 monoclonal antibody (see Material and Methods). After separation of the immunoprecipitated proteins by SDS/PAGE, they were western blotted and probed with the anti-HP1α, β, γ and mPc1 monoclonal antibodies. In an initial control experiment, where cells were transfected with an anti-βgal scFv, we found that, under these conditions, HP1β was mostly found in the insoluble fraction, with no detectable HP1β in the soluble fraction (Fig. 4A, lanes 1 and 2). However, in extracts taken from cells expressing the anti-chromodomain scFvs (372nuc, lane 3; 373nuc, lane 4), we found that HP1β

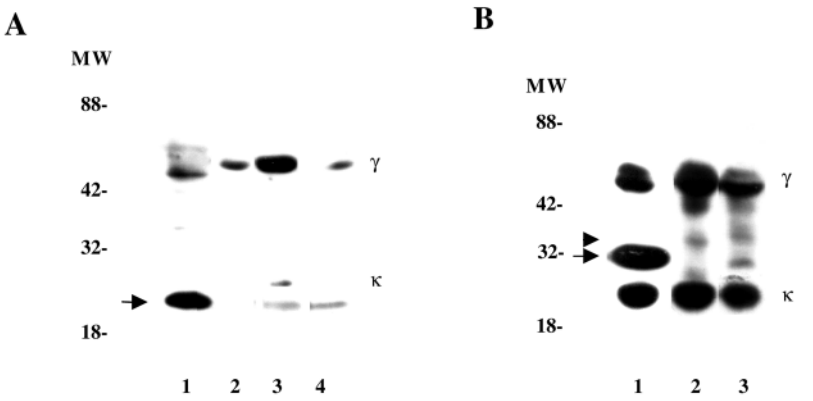

Fig. 4. In vivo interaction of anti-CD scFvs with endogenous HP1β protein. (A) COS cells were transiently transfected with DNA encoding scFv fragments and analysed after 48 hours. A western blot analysis was performed with anti-HP1β MAC 353 of the insoluble pool from COS fibroblasts transfected with anti-βgal scFv (lane 1). Western blot of soluble proteins from COS cells transfected with anti-βgal (lane 2), 372nuc (lane 3) and 373nuc (lane 4) immunopurified using anti-myc 9E10 and then probed with anti-HP1β monoclonal antibody MAC 353 is shown. The arrow points to the HP1β protein. (B) Western blot analysis of anti-βgal (lane 1), 372nuc (lane 2) and 373nuc (lane 3) scFv fragments immunopurified from transfected COS cells, viewed with anti-myc IgG. The migration of molecular weight markers (in kDa) is shown on the left and the heavy (γ) and light (k) chains are indicted on the right. The arrow points to the anti-βgal scFv fragment (lane 1) and the arrowhead points to the anti-CD scFv fragments (lanes 2 and 3).

could be detected in the soluble fraction. Thus, expression of the intracellular scFvs results in the release of HP1β from the insoluble fraction, which is most probably caused by direct in vivo binding of the intracellular scFvs to HP1β. Moreover, despite the fact that scFvs interact with all three HP1 proteins in vitro (Fig. 1) we found that the anti-chromodomain scFv fragments did not immunoprecipitate HP1α, γ and mPc1 (data not shown), suggesting that the more accessible target of the intracellular antibodies in living cells is the HP1β CD.

We also undertook additional immunoblots using the anti-myc IgG to determine the level of expression of anti-CD scFvs relative to scFvs directed against a non-relevant antigen (βgal). As shown in Fig. 4B, the intracellular anti-βgal scFv fragment (lane 1) is expressed at a higher level with respect to the anti-CD scFv fragments (lanes 2 and 3). The difference in molecular weight between the anti-βgal and the anti-CD scFv fragments (372nuc and 373nuc) is because of the presence of three NLS at the C-terminal of the anti-CD molecules. We also noted that, in addition to the weak anti-CD scFv bands, other immunoreactive bands at lower and higher molecular mass are also present (Fig. 4B, lanes 2 and 3). The lower bands represent degradation products, whereas the higher bands are probably the result of post-translational modification of the antibody fragments, such as ubiquitination (Cardinale et al., 2001). The marked reduction in the expression level is likely to reflect the general state of the transfected cells, which, as already mentioned, appear sick and some of them start to detach from the plate.

Diverting HP1β into nuclear aggregates using intracellular antibodies

We next explored the effect of intracellular scFvs expression on HP1 nuclear localization in living cells. We analyzed the effect of scFv 372nuc because of the higher transfection efficiency achieved with this plasmid, which allowed us to look at more cells. Accordingly, scFv 372nuc was transfected into mouse NIH-3T3 fibroblasts, which were chosen because they possess large Hoechst-positive heterochromatic blocks that are easily observable with a fluorescence microscope: these blocks provide a ‘landmark’ against which HP1 localization could be measured. The steady-state distributions of HP1 isoforms in NIH-3T3 cells was as expected (Wreggett et al., 1994; Horsley et al., 1996; Minc et al., 1999). HP1α and HP1β were found in large nucleoplasmic foci that largely colocalized with Hoechst-positive heterochromatic blocks (Fig. 5B,F for HP1β; Fig. 5J,N for HP1α). HP1γ labeling was different as it was distributed in multiple small dots that were mainly located outside of the nucleoli and of the Hoechst-positive heterochromatic blocks (Fig. 5R).

Strikingly, we found that expression of the anti-CD 372nuc scFv fragment in some NIH-3T3 cells resulted in the formation of distinct HP1β nuclear aggregates of different size, which were not coincident with Hoechst-positive heterochromatic blocks (Fig. 5A-D,E-H). As seen, the anti-myc antibody localizes to distinct foci (Fig. 5C,G), which are coincident with HP1β foci (Fig. 5B,F). These coincident foci do not colocalize with the Hoechst-positive heterochromatic blocks (compare Fig. 5C,G with 5A,E). We conclude that these novel nuclear aggregates consist of HP1β bound to the intracellular scFv.

We extended this study to investigate the effect of 372nuc scFv expression on the localization of HP1α, HP1γ and mPc1. In cells where expression of the intracellular antibody resulted in the formation of nuclear aggregates, the localization of HP1α was not disturbed (Fig. 5, see the nucleus in the upper middle of panels K,L). Other cells, which showed a diffuse pattern of scFv expression (Fig. 5, upper nucleus in panel O), also exhibited a diffuse HP1α signal throughout the nucleus (Fig. 5, upper nucleus in panel N). Finally, in some cells the scFv fragment localized to the typical HP1α heterochromatic foci (see lower nucleus in Fig. 5, panels M-P). The pattern of HP1γ and mPc1 distribution did not change in cells expressing the intracellular scFv (Fig. 5, panels Q-T for HP1γ and panels U-X for mPc1).

These findings are largely consistent with the immunoprecipitation experiments (Fig. 4) and support the conclusion that the anti-CD scFv fragments interact almost exclusively with the HP1β isoform in vivo. This interaction then diverts HP1β, in an antibody-specific way, from its normal physiological location (Fig. 5A-H). Whether the diffuse distribution of HP1α seen in some nuclei is caused by a direct in vivo interaction with the anti-CD scFvs or is the result of a general perturbation of chromoproteins distribution, as cells are dying (see next section), is being studied.

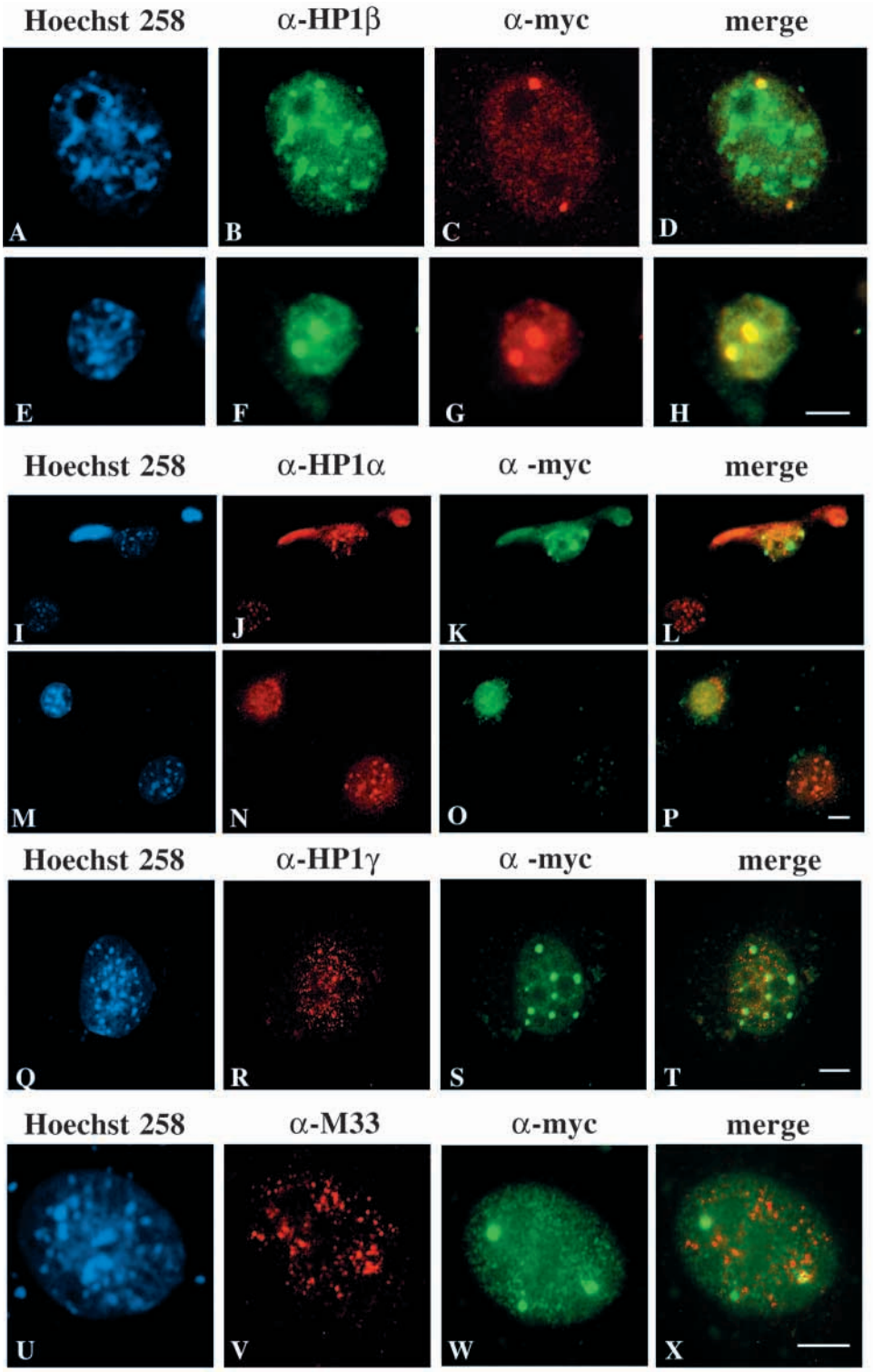

Fig. 5. Colocalization analysis of anti-CD scFvs with endogenous HP1β, HP1α, HP1γ and mPc1 (M33) proteins. Panels (A,E,I,M,Q,U) depict nuclei stained with Hoechst 33258, which preferentially stains the heterochromatin blocks present in mouse nuclei. Panels (B,F,J,N,R,V) are the same nuclei and depict the steady-state distributions of HP1 isotypes and mPc1(M33) proteins. Panels (B) and (F) represent the distribution of HP1β; panels (J) and (N) represent the distribution on HP1α; panel (R) represents the distribution of HP1γ and (V) the distribution of mPc1(M33). Panels (C,G,K,O,S,W) are the same nuclei and depict the distribution of the 372nuc scFv. Panels (D,H,L,P,T,X) represent the merged images. Scale bars, 5 μm.

Chromodomain inactivation causes cell death

We noticed that cells expressing intracellular anti-chromodomain scFvs did not thrive, with many of the cells exhibiting cell shrinkage, which is a feature of apoptosis. In order to explore the cytotoxic effect of the intracellularly expressed anti-chromodomain antibodies, we decided to quantify the number of cells that undergo cell death by using two markers of apoptosis. First, we used Annexin V, which detects alterations at the level of the plasma membrane. Second, we used the blue-fluorescent Hoechst 33342 dye, which stains the condensed chromatin of apoptotic cells more brightly than the chromatin of non-apoptotic cells. On the basis of the combined staining patterns of these dyes, we were able to distinguish between normal, apoptotic and dead cells.

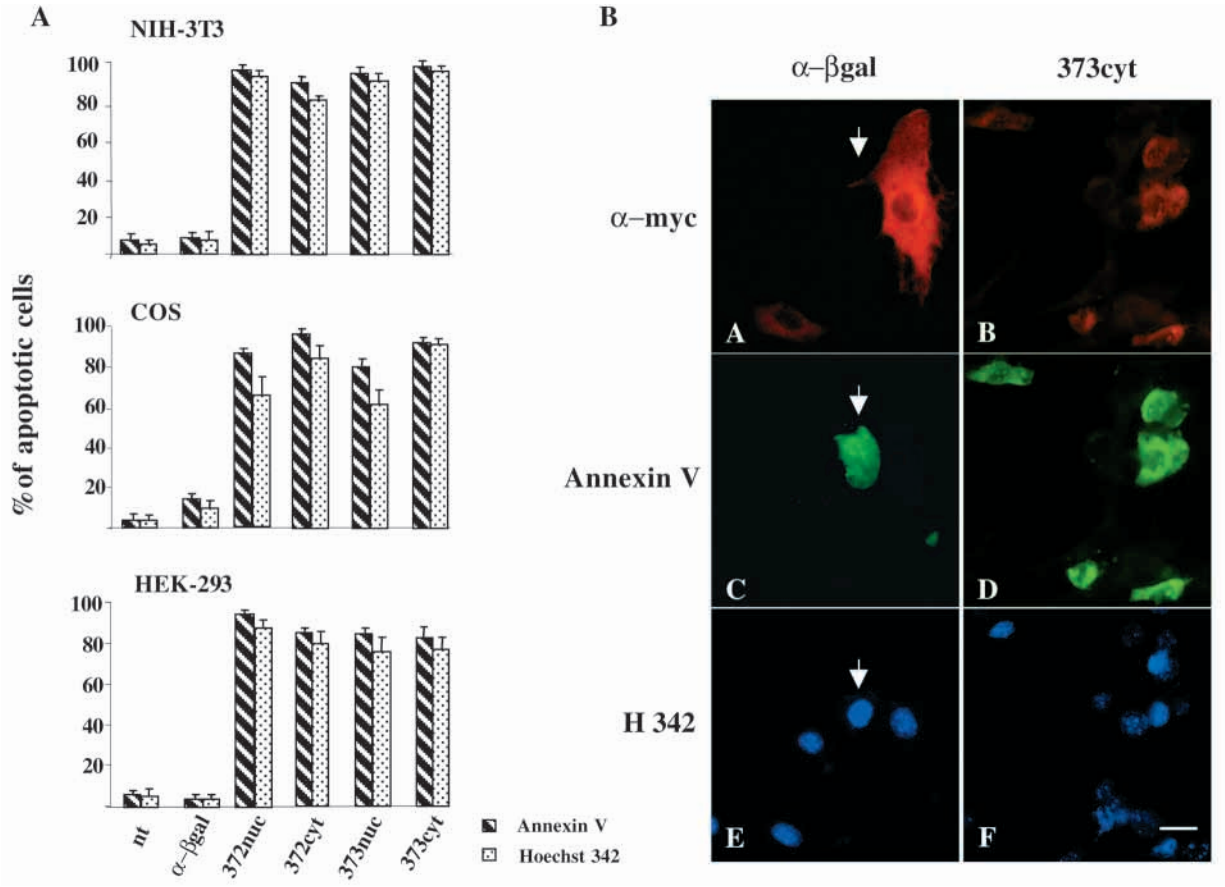

Fig. 6. Anti-CD scFvs induce cell death in mammalian cells. (A) The number of Annexin-V- and Hoechst-33342-positive cells were counted in non-transfected cell (nt) population and cells transfected with different scFv fragments (as indicated). Each histogram shows the results obtained by transfection of different cell lines as indicated. The figure shows the average of three different experiments for each cell line used. At least 60 positively transfected cells for each plasmid were counted. (B) The left hand column shows a field of cells transfected with the anti-βgal scFv and then stained with Annexin V and Hoechst 33342. Arrow points to a single Annexin-V/Hoechst-33342-positive cell that is not transfected. The right hand column shows a field of cells transfected with the 373cyt scFv. Many more cells were positive for Annexin V and Hoechst 33342. Scale bars, 20 μm.

We transiently transfected mouse NIH-3T3, simian COS fibroblasts and human HEK-293 cells and determined the phenotype of transfected cells using the two markers of apoptosis. As seen in Fig. 6A,B the percentage of apoptotic cells varies between 80-90% in COS and HEK-293 and 95-98% in mouse NIH-3T3 fibroblasts, when calculated on the basis of Annexin-V-positive cells, and between 70-90% on the basis of Hoechst-33342-positive cells. Only very few Annexin-V/Hoechst-positive cells are also positive for propidium iodide (not shown), indicating that the cells examined are not necrotic but are actually undergoing a process of programmed cell death. The cytoplasmic versions of the anti-CD scFvs are equally as efficient in inducing apoptosis as the nuclear scFvs (Fig. 6). This observation suggests that the cytoplasmic scFvs interact with HP1 proteins in the cytoplasm and that this interaction may interfere with their normal intracellular traffic. By contrast, much lower levels of apoptosis were observed in cells transfected with the anti-βgal scFv constructs.

Cell death induced by anti-CD scFv is p53 independent and is only partly prevented by Z-VAD fmk treatment

We next investigated whether the cell death seen in our experiments was dependent on p53 tumor-suppressor gene activity because p53 is activated in response to a variety of signals of DNA damage (Levine, 1997). Human osteosarcoma Saos-2 cells (Chen et al., 1990), which are null mutants for p53, were transiently transfected with the different anti-CD scFv fragments. Fig. 7A shows that over 90% of Saos-2 cells transfected with 372nuc or 373nuc plasmid undergo cell death, suggesting that cell death occurs in a p53 independent pathway. In the control experiments, where Saos-2 cells were transfected with an irrelevant anti-βgal scFv fragment or were not transfected, only 10% of the cells undergo apoptosis.

The role of caspases (cysteine proteases cleaving after particular aspartate residues) in anti-CD scFv-dependent apoptosis was investigated by treating cells with Z-VAD fmk, a wide spectrum inhibitor of caspases (Ekert et al., 1999). We therefore treated NIH-3T3 cells with Z-VAD fmk after transfecting them with 372nuc and 373nuc scFv fragments. As seen in Fig. 7B there was a small, but significant effect of Z-VAD fmk on cell death that results from the expression of anti-CD scFvs (around 20-25% decrease of cell death). By contrast, the caspase inhibitor is able to rescue more than 50% of non transfected cells treated with 250 μM H_2O_2. The cytoplasmic version of the two anti-CD scFv fragments was equally

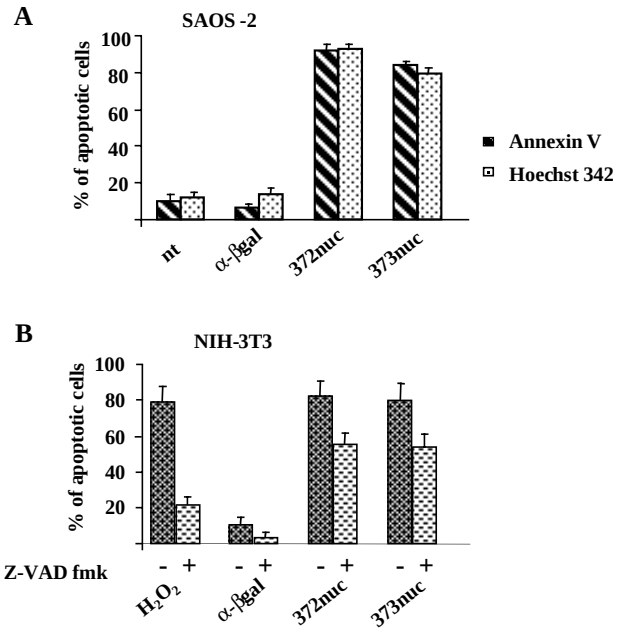

Fig. 7. Anti-CD scFvs induce p53-independent cell death, which is partly prevented by Z-VAD fmk treatment. (A) Non-transfected cells (nt) and cells transfected with anti-βgal, 372nuc and 373nuc scFv fragments (as indicated) were counted for their positive staining with Annexin V and Hoechst 33342. The figure shows the average of three different experiments. At least 60 positively transfected cells for each plasmid were counted and checked. (B) Hoechst-33342-positive cells were counted from non-transfected cells incubated with H_2O_2 and cells transfected with different scFvs (as indicated) in the presence or in the absence of the caspase inhibitor Z-VAD fmk.

effective in inducing cell death when transfected into Saos-2 $p53^{-/-}$, and this effect was only partly rescued by Z-VAD fmk treatment in NIH-3T3 cells (data not shown).

We conclude that the anti-chromodomain intracellular antibody fragments induce cell death via a pathway that is p53 independent and only partly caspase dependent.

Discussion

An increasing number of studies have indicated a fundamental role for mammalian chromodomain proteins in genome organization and expression (Jones et al., 2000). In this paper we show that expression of anti-CD intracellular scFv fragments in cells is lethal.

Despite the broad in vitro specificity of the scFvs, immunoprecipitation experiments revealed that the scFvs in vivo target was more limited and probably restricted to HP1β (Fig. 4). This was supported by subnuclear localization studies, which showed that, just before transfected cells begin to exhibit signs of apoptosis/cell death, HP1β was diverted from its normal nuclear localization into intranuclear aggregates (Fig. 5A-H). Diversion from typical steady-state nuclear localization was not observed for HP1α, M32 (HP1γ) or mPc1, which contain highly homologous, but non-identical, CDs. Nevertheless, we cannot rule out the possibility that other as yet unidentified CD proteins might be recognized by the anti-CD scFvs, and the inactivation of CD could contribute to the observed cell death phenotype.

The nuclear aggregates observed (Fig. 5A-H) closely resemble previously described cytoplasmic aggresomes that result from the intracellular expression of scFvs. These cytoplasmic aggresomes contain ubiquitinated scFv fragments as a complex with trapped antigen (Cardinale et al., 2001): the anti-chromodomain scFvs also appear to contain modified, possibly ubiquitinated, antigen-antibody complexes (Fig. 4B). The formation of nuclear aggregates are consistent with our previous work, which has suggested that intracellular scFvs act by interfering with intracellular traffic of target proteins, leading to mis-localization away from their normal physiological sites of action (Lener et al., 2000). The efficacy of the cytoplasmic scFvs can also be explained by a similar mechanism where newly synthesized HP1 molecules are inhibited or slowed down in their translocation towards the nucleus as they become bound by the scFvs (Fig. 5). Studies presently underway to characterize the intranuclear aggregates described in this paper will shed light on their possible similarities with cytoplasmic aggresomes, that is, to verify whether they are ubiquitinated and whether they are associated with nuclear proteasomes.

Clearly defined nuclear aggregates are not visible in all cells. In some cells the anti-chromodomain scFvs and HP1 proteins are diffuse, and a widespread fluorescence signal is distributed throughout the nucleoplasm (Fig. 3C; Fig. 5O). We suspect that this uniform distribution is an indirect effect of cell death, because it appears mostly in cells that present early signs of apoptosis, as determined by Annexin V staining. Moreover, immunolocalization of HP1 proteins in cells undergoing apoptosis as a result of exposure to H_2O_2 show a significant delocalization of these proteins in the nucleoplasm (I.F. and S.B., unpublished).

The in vivo preference of anti-chromodomain scFvs for HP1β may reflect the fact that the epitope in the HP1β chromodomain is simply more accessible to the scFvs in bulk chromatin. In addition, the preference may reflect the observation that HP1β shows dynamic changes in nuclear localization during the cell cycle (Minc et al., 1999; Kourmouli et al., 2000), and it is this dynamic behavior that allows the intracellular scFvs to bind and sequester the HP1β in the nuclear aggregates. Recent work using isotype-specific antibodies showed that, despite their being similar in their primary sequence, HP1 isotypes segregate into distinct nuclear domains (Minc et al., 1999). In particular, during mitosis, HP1β detaches from heterochromatin, becomes cytoplasmic and only later re-assembles onto the polar surfaces of anaphase chromosomes (Kourmouli et al., 2000). To a lesser degree, HP1γ also shows dynamic behavior during the cell cycle; HP1α largely remains on the chromosomes (Minc et al., 1999; Kourmouli et al., 2000).

What physiological function of HP1β is perturbed by the intracellular scFvs and thus leads to the dramatic cell death phenotype? One possibility is that the intracellular antibodies hinder the assembly and/or maintenance of heterochromatin complexes. The perturbation of such complexes could lead to a variety of lesions, each of which is likely to be incompatible with cell survival. They include activation of normally silent genes (Jones et al., 2000), defects in centromere function (Kellum and Alberts, 1995), ectopic telomere fusions (Fanti et al., 1999) and inappropriate NE re-assembly (Kourmouli et al., 2000). In particular, we note that HP1 proteins interact with

the non homologous end joining (NHEJ) protein Ku70 (Song et al., 2001), which has been found to play an important role as part of a complex with Sir3 and Sir4 in maintaining genome integrity in budding yeast (Guarente, 1999). By analogy, mammalian HP1 proteins may serve a similar role to Sir3 and Sir4 in maintaining structured chromosomal domains that are necessary for cell survival. Interference with this function could lead to catastrophic effects on genome integrity. Another site where perturbation of HP1 function is likely to have a dramatic effect is at the centromere. The centromere is known to harbor proteins that are necessary for cell survival. The best known being *survivin* (Li et al., 1998; Li et al., 1999). Loss of survivin activity leads to irreversible cell death, and we suspect that anti-chromodomain scFvs may activate this pathway. Whatever the mechanism, our results provide evidence that scFvs directed against distinct epitopes of the HP1 chromodomain induce cell death when expressed in mammalian cells, and this result strengthens the hypothesis that there is an absolute requirement for HP1 chromodomain function in mammalian cells.

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