Characterization of the cytotoxic effect of a chimeric restriction enzyme, H1⁰-FokI

Research Article

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Key words: Chimeric nuclease, cytotoxic, apoptosis

Abbreviations: calcein-AM, (CN-AM); diamidino-2-phenylindole dihydrochloride, (DAPI); Drosophila Ultrabithorax, (Ubx); ethidium homodimer, (ET-HD); fluorescein isothiocyanate, (FITC); fluorescence activated cell sorter, (FACS); green fluorescent protein, (GFP); phosphate buffer saline, (PBS); Polymerase Chain Reaction, (PCR); post-transfection, (PT); propidium iodide, (PI)

This publication was made possible by NIH Grant Number RR016476 from the MFGN INBRE Program of the National Center for Research Resources.

Received: 13 April 2006; Accepted: 19 April 2006; electronically published: May 2006

Summary

Our primary goal was to create an efficient cytotoxic agent. To do this, we created a gene that expresses a chimeric hybrid of the linker histone, H1⁰ and the nuclease domain of the type IIs restriction enzyme, FokI. The linkage of the FokI nuclease domain to a high affinity but low DNA-sequence-specificity binding protein is unique. It is highly cytotoxic. We demonstrate, by transiently transfecting 3T3 mouse fibroblasts, that 63% of the cells taking up the chimeric gene are killed. The chimeric protein is localized to the nucleus. An extract of the protein produced in E. coli degrades DNA, indicating that it is nucleolytically active. The ultimate mechanism through which the chimeric protein produces cell death is likely through the induction of apoptosis.

I. Introduction

The H1, or linker, histones are a well characterized, multivariant family of small basic proteins that play a major role in chromatin organization (Ramakrishnan, 1997 and Widom, 1998). They bind to chromatin with a high affinity (Mamoon et al, 2002) and at a ratio of up to one or more per nucleosome (Widom, 1998; Simpson, 1978). Recently, it was demonstrated that H1 histone to which the large green fluorescent protein (GFP), is fused at its carboxy-terminal domain, can enter the nucleus, where it appears to bind nucleosomes normally (Lever et al, 2000; Misteli et al, 2000). Experiments conducted with the H1-GFP revealed that H1-GFP binding to chromatin is quite dynamic with a residence time on the order of a minute. These observations suggest that the H1 linker histones would be ideal candidates for construction of fusion proteins that will enter cell nuclei. A potentially useful protein to deliver to the nucleus would be a nuclease to act as a cytotoxic agent. Due to the chromatin binding properties of H1 histone, using it to direct a nuclease to the nucleus of a living cell could well prove to have sufficient cytotoxicity as to be of clinical usefulness.

The nuclease domain of the type IIs FokI restriction-modification enzyme has been used as a source of a nuclease for the construction of chimeric restriction enzymes (Kim and Chandrasegaran, 1994; Huang et al, 1996; Kim et al, 1996, 1997, 1998; Kim and Pabo, 1998). The type IIs FokI restriction-modification enzyme was originally characterized by Kita et al, in 1989. It is useful for the construction of chimeric restriction enzymes because its recognition domain is separate from its cleavage domain. Several chimeric restriction enzymes have been engineered in which the DNA recognition domain of one protein has been fused to the endonuclease domain of FokI. These reports include the linking of Drosophila Ultrabithorax (Ubx) homeodomain to the cleavage domain of FokI restriction endonuclease (Kim and Chandrasegaran, 1994). This group also reported the creation of a novel site-specific endonuclease by linking the N-terminal 147 amino acids of yeast Gal4 to the cleavage domain of FokI endonuclease (Kim et al, 1998). The fusion protein was found to be active and under
optimal conditions, bound to a 17 bp consensus DNA site and cleaved near this site. Subsequent reports described the engineering of a zinc-finger-FokI restriction endonuclease and characterization of its DNA cleavage specificities (Huang et al, 1996; Kim et al, 1996, 1997 and Kim and Pabo, 1998). These chimeric nucleases have also been used in several application related studies. For example, a zinc-finger-FokI nuclease was used to cause DNA cleavage and mediate homologous recombination between DNA sequences in Xenopus laevis oocyte nuclei (Bibikova et al, 2001). Similarly, the zinc-finger-FokI chimeric nuclease was also used to stimulate gene targeting in human cells (Porteus and Baltimore, 2003) and in Drosophila (Bibikova and Beumer, 2003). Beyond the use of FokI chimeras to stimulate homologous recombination, the cytotoxicity of these chimeras has yet to be evaluated. The linkage of the FokI nuclease domain to H1 would generate a potent nuclease with superior nuclear localization affinity and avid DNA binding potential. Furthermore, since the desired outcome is to generate lethal levels of DNA cleavage, the low sequence preference of H1 (Wellman et al, 1994; Wellman et al, 1999; Mamoon et al, 2002; Renz, 1975 and Marekov and Beltchev, 1978) with its relatively short residence time (Lever et al, 2000; Misteli et al, 2000) makes it an ideal candidate to reputedly deliver a nuclease to the proximity of DNA. In this paper we demonstrate the efficacy of this idea by using a transient transfection system in which an H1 linker histone is used to deliver sufficient nuclease activity to cells, to be lethal.

II. Materials and methods

A. Cloning of the vector DNA constructs

The 588 bp FokI endonuclease domain was amplified by Polymerase Chain Reaction (PCR) of the plasmid, pUC 19/FokI (ATCC) using two primers, UNcoFokI (5’-CCATGGTGTACGATAGCAC-3’) and DBamHI_FokI (5’-GGATCCATTAAAGTTATCTGACC-3’) [all primers were from Integrated DNA Technologies, Coralville, Iowa, U.S.A.] carrying Nco I and BamH I sites respectively. The PCR cycle was [95°C, 5 minutes; 95°C, 1 minute; 46°C, 1 minute; 72°C, 1 minute)x5 cycles and [95°C, 1 minute; 54°C, 1 minute; 72°C, 1 minute)x15cycles]. The Nco I and BamH I (all cloning enzymes were from New England Biolabs) digested PCR product and 4904 bp pBSIIKS(-)/H1° (kindly given by Brown, D.T., University of Mississippi Medical Center, Jackson, Mississippi 39216, U.S.A.) vector DNA were ligated to form the 4144 bp pBSIIKS(-)/H1°-FokI. Next, the cohesive and blunt ends generated by the sequential Stu I and BamH I digestion of the 3578 bp PBSIIKS (-)/H1° and the 4144 bp pBSIIKS(-)/H1°-FokI INTO vectors were ligated in a two-step reaction. Using this scheme, the sequence of H1° was fused in frame (with the insertion of a GCC triplet coding for alanine between the junctions of the two sequences) to the cleavage domain of FokI, to form the 4184 bp pBSIIKS(-)/H1°-FokI. For the subcloning of FokI endonuclease, the 4184 bp pBSIIKS(-)/H1°-FokI was digested with Nco I and BamH I enzymes. This yielded the 2988 bp pBSIIKS(-) vector, 588 bp H1° and 602 bp FokI cleavage domain. The 2988 bp pBSIIKS(-) vector and 588 bp FokI insert DNA were ligated to form the 3596 bp pBSIIKS(-)/FokI. The construction of vector DNA sequences used for transfection, immunofluorescence, western blot and protein purification experiments was done by similar strategies (Dissertation, Alam Naved, University of Mississippi Medical Center, Jackson, Mississippi 39216, U.S.A.). The primary constructs pertinent to this work are shown in Figure 1.

B. Transient transfection of the mouse cells with DNA sequences

Balb/c 3T3 mouse fibroblasts (clone A31) from American Type Cell Culture (Manassas, Virginia, U.S.A.) were grown and maintained as previously described (Brown et al, 1996). Approximately 5.5x10⁶ cells plated in a 60 mm culture dish (Coming Inc., Corning, New York, U.S.A.) were grown to 70% confluence. 2 µg of plasmid DNA was suspended in Dulbecco’s Modified Eagle Medium (Invitrogen, Carlsbad, California, U.S.A) [without serum and antibiotics] to a total volume of 150 µl. 15 µl of the Polyfect transfection reagent (Qiagen Inc., Valencia, California, U.S.A.) was added to the DNA solution and the DNA-reagent solution was mixed by pipetting up and down 5 times. The mixture was incubated at room temperature for 10 minutes. During the incubation period, cells were washed twice with fresh media to remove dead cells. Three ml of the media was added to cells. After the incubation period, 1ml of media was added to the DNA-reagent solution and it was mixed by pipetting twice. The DNA solution was then added to the cells with gentle shaking of the culture dish. The cells were incubated for expression of the recombinant gene and the transfectants were analyzed between 24-72 hours post-transfection (PT).

C. Staining of the mouse fibroblasts with calcine-AM and ethidium-homodimer cell viability indicator dyes

The cells transfected in single-well Lab-Tek II Chamber Side System (Nalgene Nunc Int., Rochester, New York, U.S.A.) were washed twice with PBS, 48 hours PT and the chamber well was removed to prepare the cells for dye staining. A combination of 2 µM calcine-AM (CN-AM) and 4 µM ethidium homodimer (ET-HD) viability-indicator dyes (Live/Dead Cell Viability Assay Kit from Promega, Madison, Wisconsin, U.S.A.) was used to stain the cells and they were covered with a cover-slip for observation. A fluorescence microscope equipped with a low magnification Nikon Zeiss (Melville, New York, U.S.A.) lens was used to visualize the cells within 5 minutes of staining them with the dyes. The CN-AM and ET-HD fluorescence was observed using a Fluorescein isothiocyanate (FITC) and a Texas Red filter respectively.

D. Immunofluorescence staining of the mammalian transfectants

The cells transfected in single-well Lab-Tek II Chamber Side System (Nalgene Nunc Int., Rochester, New York, U.S.A.) were washed twice with phosphate buffer saline (PBS), 48 hours PT and the chamber wells were removed for further treatment. Following cell fixation in 4% formaldehyde/PBS for 10 minutes, the cells were treated with 0.5% Triton-PBS for 5 minutes to permeabilize the cell membranes. The cells were washed thrice with PBS and non-specific binding sites for the antibody were reduced by incubating the cells in 10% goat sera/PBS for 30 minutes at 37°C. The cells were then incubated with a 1:100 dilution of anti-my antibody (c-Myc 9E10 from Santa Cruz Inc., Santa Cruz, California, U.S.A.) for 1 hour at 37°C. Thereafter, the cells were washed twice with PBS and they were incubated with a 1:400 dilution of anti- mouse IgG-FITC antibody (Santa Cruz Inc., Santa Cruz, California, U.S.A.) from goat at 37°C. After 1 hour, the cells were washed thrice with PBS and treated with 300 nM solution of 4’6-diamidino-2-phenylindole dihydrochloride (DAPI) dye (Molecular Probes, Carlsbad, California, U.S.A.) for 5 minutes to stain the DNA of nuclei. A drop of anti-fade reagent (Molecular Probes, Carlsbad, California, U.S.A.) was added to
the slide to preserve the fluorescence intensity and the cells were covered with a cover-slip. DAPI staining of the cells was observed with a DAPI filter using a fluorescence microscope while the fluorescence from the myc-tagged protein was observed with a FITC filter.

E. Western blot analysis of whole cell lysates from the mammalian transfectants

Cells were grown and transfected in 75 cm² culture flasks and were washed thrice with PBS, 72 hours PT and harvested using a cell scrapper. They were suspended in 1 ml lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 2 mM EDTA, complete EDTA-free protease inhibitor cocktail tablet] for lystate preparation. The cell lystate was sheared by passing through a 23G syringe, centrifuged at 10,000 g for 10 minutes and the protein-rich supernatant was collected. An aliquot of the lystate was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were transferred to a nylon membrane (Biorad, Hercules, California, U.S.A.) and non-specific binding sites for the antibody were reduced by incubating the membrane in 10% goat sera/PBS for 1 hour at room temperature. The membrane was then incubated with a 1:100 dilution of anti-myc antibody (c-Myc 9E10 from Santa Cruz Inc., Santa Cruz, California, U.S.A.) for 1 hour at room temperature. After three washes with PBS, the membrane was further incubated with a 1:5000 dilution of anti-mouse IgG-HRP antibody (Pierce Inc., Rockford, Illinois, U.S.A.) from goat at room temperature for 1 hour. Detection of the myc-tagged protein was performed using the recommended protocol of Pierce Inc. and the signal was recorded on Eastman Kodak (Rochester, New York, U.S.A.) Biomax ML X-ray film.

F. Quantitative determination of the cell viability of transfectants by propidium iodide staining

The cells transfected in 60 mm culture dishes were harvested 72 hours post transfection, washed twice with PBS and suspended in 1 ml of fresh PBS. The cells were stained with (100 μg/ml) propidium iodide (PI) dye [Sigma Aldrich Corp., St. Louis, Missouri, U.S.A] and analyzed by flow cytometry within 5-10 minutes of dye staining. 10,000 events were captured by a Coulter SC500 (Beckman Coulter, Fullerton, California, U.S.A.) for one hour at room temperature. The intensity of PI staining was subtracted from the final calculation with respect to forward light scatter of the cells. The background staining for PI staining was subtracted from the final calculation for the determination of cell viability for each transfectant. The necrotic control cells were prepared by treatment with methanol for 10 minutes at -20°C.

G. Annexin V and propidium iodide staining of the mammalian transfectants

The cells transfected in 60 mm culture dishes were washed thrice with PBS, 72 hours post-transfection and harvested in the same solution with the aid of a cell scrapper. They were collected by centrifugation at 10,000 g for 30 seconds at 4°C. The cell pellet was re-suspended at 1 x 10⁶ cells/ml in 1X annexin-binding buffer (all assay reagents were from Molecular Probes, Carlsbad, California, U.S.A). Next, 5 μl of annexinV alexa fluor 488 dye and 1μl (final concentration of 100 μg/ml) of PI dye were added to a 100 μl aliquot of the cells. The cells were then incubated in dark for 15 minutes. 400 μl of 1X annexin binding buffer was added to the cells and they were mixed briefly and stored at 4°C. The cells were analyzed by flow cytometry within 10 minutes of the protocol’s final step. A population of cells was induced to undergo apoptotic death by a combination of serum-starvation for 72 hours and treatment with the apoptosis-inducing drug, camptothecin (10 μM in dimethyl sulfoxide) [both reagents were from Invitrogen Inc., Carlsbad, California, U.S.A] for 12 hours (inclusive of the 72 hours serum-starvation period) before the harvesting of cells for analysis.

H. In vitro assay for the cleavage of DNA by purified proteins

Rosetta DE3 plys S cells (Novagen, San Diego, California, U.S.A.) were co-transformed with various pET16b vector constructs along with pACYC177 (kindly provided by Dr. S. Chandrasegaran, Department of Environmental Health Sciences, The Johns Hopkins University, Baltimore, Madison, 21205, U.S.A.). A single colony of transformed cells was used for the preparation of protein for a 1L culture. Production of the protein from bacteria and its purification using metal-chelate chromatography was performed according to instructions from the fifth edition of the Qiagen (Valencia, California, U.S.A.) manual. Plasmid pUC 18 DNA (New England Biolabs, Ipswich, Massachusetts, U.S.A.) was incubated with equal concentrations of E.coli produced purified proteins in DNA cleavage buffer [75 mM KCl, 10 mM MgCl₂, 10 mM Tris-Cl; pH 8.0, 3 mM DTT, 5% glycerol, 100 μg/ml of E. coli tRNA and 50 μg/ml bovine serum albumin]. The digestion reaction was for 4 hours at 37°C followed by analysis of the products by electrophoresis on ethidium bromide (0.5 μg/ml) stained 0.7% agarose gel.

III. Results

A. The introduction of H1°-FokI chimeric DNA in mouse fibroblasts is cytotoxic

We transiently transfected mouse 3T3 fibroblasts with the sequences coding for the H1°-FokI hybrid (Figure 1, materials and methods) in order to drive abundant expression of the H1°-FokI hybrid off of the strong CMV promoter. Viability was assessed by staining of the cells with a combination of calcien-AM and ethidium homo-dimer dyes. This dual color assay, which discriminates between live and dead cells, was used to observe differences in staining pattern of H1°-FokI transfected and control cells.

As shown in Figure 2, it is evident that by 72 hours, cells that are transiently transfected with the H1°-FokI hybrid construct show significantly more cell death than cells mock-transfected or transfected with control vectors carrying only the H1° or the FokI cleavage domain. The only cells showing significant cell death other than those transfected with the H1°-FokI hybrid construct were the control cells killed with methanol.

B. The H1°-FokI protein re-localizes from the cytoplasm to the nucleus in the transfected cells

To verify that the H1°-FokI hybrid localized to the nucleus as predicted and as indicated by the cytotoxic effect of transfection with an H1°-FokI hybrid expressing vector, we subcloned the H1°-FokI hybrid, the H1° and the FokI nuclease coding regions into a pcDNA3.1(-)myc-HisA (Invitrogen Inc. Carlsbad, California, U.S.A.) vector such that they would carry a myc tag on their carboxy termini upon expression (materials and methods). Mouse 3T3 fibroblasts were transiently transfected and the location of the myc-tagged proteins were visualized by

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immunofluorescent-staining to the myc antigen as described in materials and methods. These results are shown in Figure 3. As expected, H1º localizes strictly to the nucleus. The FokI nuclease domain by itself contains no DNA binding, recognition or nuclear localization capacity and accordingly remains in the cytoplasm. The H1º-FokI hybrid is as predicted also found in the nucleus although some is also seen in the cytoplasm in some of the cells.

C. Western blot analysis of cell lysate from transfected mouse cells

A western blot of extracts of these transfectants, using antibodies to the myc tag, demonstrates that the transiently expressed proteins are the expected full length (Figure 4) and the cell viability results are not due to the breakdown of any of the transfectant products. The myc-tagged H1º protein migrates with an apparent mobility of 37 kD although the protein has a calculated molecular weight of approximately 22 kD. This altered mobility on SDS-PAGE gels is well described (Welch and O’Rand, 1990; Kasinsky et al, 2001; Nicholson et al, 2004) and is due to the presence of highly positively charged lysine and arginine residues in the carboxy terminal tail. Likewise, the myc-H1º-FokI protein exhibits a mobility deviation from the predicted molecular weight of roughly 44 kD. The myc-FokI protein, lacking the H1º component, migrates as expected on the gel.

Figure. 1. Cloning of the H1º-FokI restriction enzyme system. (A). Schematic of vector constructs and the DNA sequences of the crucial junctions of H1º, FokI and H1º-FokI. (B). Restriction analysis of plasmid constructs, resolved on a ethidium bromide (0.5μg/ml) stained 0.7% agarose gel. Lane 1. DNA Marker with sizes as indicated, Lane 2. pBSIIKS(-), Lane 3. XbaI + BamHI pBSIIKS(-), Lane 4. pBSIIKS(-)/ H1º, Lane 5. XbaI + BamHI pBSIIKS(-)/H1º, Lane 6. pBSIIKS(-)/ FokI, Lane 7. XbaI + BamHI pBSIIKS(-)/ FokI, Lane 8. pBSIIKS(-)/ H1º-FokI, Lane 9. XbaI + BamHI pBSIIKS(-)/ H1º-FokI.
Figure 2. Staining of mammalian transfectants with cell viability indicator dyes for determination of viability by fluorescence microscopy. Live cell column shows cells, as labeled in rows, stained with the calcein-AM dye. Dead cell column shows cells stained with the ethidium homo-dimer dye.
**Figure 3.** Cellular localization of recombinant proteins after transient transfection. Column 1 (FITC): Recombinant proteins detected by immuno-detection with an anti-myc antibody and anti-mouse IgG1-FITC antibody in cells, 72 hours post transfection with recombinant clones as labeled for each row. Column 2 (DAPI): Nuclear DAPI staining of cells, as labeled, shown in column 1.

**Figure 4.** Western blot analysis of total cell lysates from mouse fibroblast transfectants. Cells were transfected with individual DNA constructs and total cell lysate was prepared from the transfected cells, 72 hours post-transfection. The protein sample was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were transferred to a nylon membrane and Western blot analysis of the proteins was done. Detection of the signal from myc-tagged proteins was done using a combination of anti-myc antibody and an anti-mouse IgG1-HRP antibody from goat at room temperature for 1 hour. The myc-tagged proteins were detected in the lysate and their mobility on the SDS-PAGE was examined as discussed in the text.
D. Quantitation of H1°-FokI’s effect on cell viability

To quantify the efficiency of killing by transient transfection of cells with the H1°-FokI hybrid, we performed fluorescence activated cell sorter (FACS) analysis (materials and methods) of cells transfected with the H1°-FokI construct or with appropriate controls and stained with the cell viability indicator propidium iodide dye (Figure 5). Quantitation of these results is shown, both as a time series between 24-72 hours post-transfection, (Figure 5A) and as the average of three independent transfection experiments at 72 hours post-transfection (Figure 5B). Mock transient transfection of cells indicates that the procedure itself results in approximately 18% cell death. Controls of mock-transfected, vector alone, H1°-expressing, or the FokI-nuclease-domain-expressing, each show a comparably low rate of cell death. Only cells transfected with the H1°-FokI hybrid-expressing vector show a significant level of cell death beyond that of the transfection controls, 44%. Control cells killed with methanol show a high death rate of approximately 96%, confirming the assay’s ability to detect cell death. We demonstrated an approximately 70% transfection efficiency in the protocol used in these experiments (data not presented). Knowing that the procedural death rate is 18% (independent of transfection) and that, of the 10,000 cells measured in each FACS analysis, after 72 hours 70% are transfected, the death rate due to transfection can be calculated. The raw data indicates that 5,580 cells are alive and 4,420 cells are dead in the H1°-FokI hybrid transfectants. The death due to expression of the H1°-FokI hybrid can only arise from the 7,000 that are transfected, so the calculated death rate due to transfection with the H1°-FokI hybrid is approximately 63%.

These observed differences in the cell viability of H1°-FokI transfected cells and controls were not attributable to differences in the transfection efficiency of the cells with exogenous DNAs themselves. The transfection efficiency of cells with various plasmid constructs was roughly equal (± 5%) (data not shown). Moreover, the differences in cell viability of the transfecants were not due to any other accessory factors, such as differential culture growth conditions of cells; comparison of the FACS profiles of the H1°-FokI transfected cells with the controls in the absence of PI dye showed similar FACS scatter pattern. Differences were only observed upon the addition of the dye.

E. The H1°-FokI protein is functionally active and cleaves DNA in vitro

Because in vivo detection of DNA cleavage in 3T3 cells may be problematic, especially if an apoptotic cell death is induced, which in itself leads to DNA cleavage (Arends et al, 1990; Wylie et al, 1992), we decided to see if bacterially produced H1°-FokI hybrid protein had nuclease activity. The H1°-FokI hybrid and the H1° and FokI cleavage domains were subcloned into the his-tag containing pET16b (Novagen, San Diego, California, U.S.A.) bacterial expression vector. The proteins were expressed and enriched over a nickel column as described in materials and methods. These enriched extracts were then incubated with plasmid DNA. As shown in Figure 6, only the extract containing the H1°-FokI hybrid protein shows significant DNA degradation. Extract from non-
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Figure 6. The H1°-FokI protein is functionally active and cleaves DNA in vitro. Plasmid pUC 18 DNA was incubated with varying concentrations of E.coli produced proteins in DNA cleavage buffer [75 mM KCl, 10 mM MgCl₂, 10 mM Tris-Cl, pH 8.0, 3 mM DTT, 5% glycerol, 100 µg/ml of E. coli tRNA and 50 µg/ml bovine serum albumin]. The digestion reaction was performed for 4 hours at 37°C followed by analysis of the digestion products by electrophoresis on a ethidium bromide (0.5µg/ml) stained 0.7% agarose gel. Plasmid DNA degradation was specifically associated with purified H1°-FokI protein. pUC18 vector DNA was degraded to low molecular weight species by H1°-FokI protein purified from E.coli but not by either H1° or FokI protein.

transformed bacteria and from bacteria transformed with pET16b vector alone, pET16b with H1°, or pET16b with the FokI cleavage domain, showed no significant degradation. This suggests that the H1°-FokI protein is an effective nuclease in vitro. Because we saw little enrichment of these bacterially produced proteins after SDS gel electrophoreses and Coomassie staining, we confirmed their presence in the extracts by western blot analysis (Figure 7). The his-tagged proteins were detected and showed appropriate mobility on the SDS-PAGE gel.

F. Fluorescence microscopy visualization of features associated with cell death in the transfected fibroblasts - H1°-FokI, is cytotoxic via an apoptotic pathway

The data indicate that the H1° component of the H1°-FokI hybrid can carry the FokI nuclease domain to the nucleus where nuclease activity of the H1°-FokI hybrid leads to cell death. We studied the cells stained with the annexin V alexa fluor 488 and PI dyes using fluorescence microscopy which allowed us to discriminate between early apoptotic, late apoptotic and necrotic modes of cell death.

Typical results obtained by studying the stained cells using fluorescence microscopy are shown in Figure 8. As expected, the un-transfected live cell population showed negligible annexin V or PI dye staining. This is because in viable cells, the membrane lipids to which annexin binds, are localized on the interior side of the plasma membrane, leading to negligible staining. Viable cells also stain negligibly with PI because the uncompromised nuclear membranes restrict access of the dye to DNA. This live cell staining pattern was also seen with other controls (vector, reagent, H1° or FokI transfected cells). Necrotic cells were generated by treatment with methanol. They stained positively with both the annexin V and PI dyes. The staining pattern of the H1°-FokI transfected cells was similar to the apoptotic, camptothecin treated control cells. The H1°-FokI hybrid cells and the apoptotic control cells show a higher percentage of annexin V staining which is indicative of apoptosis.

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Figure 7. Western blot analysis of the bacterially expressed his-tagged proteins. Bacterially produced proteins were enriched for his-tagged proteins by metal-chelate chromatography. The protein sample was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were transferred to a nylon membrane and Western blot analysis of the proteins was done. Detection of the signal from the his-tagged proteins was done using a combination of anti-his antibody and an anti-mouse IgG-HRP antibody from goat at room temperature for 1 hour. The his-tagged proteins were detected in the sample and their mobility on the SDS-PAGE was examined as discussed in the text.

Figure 8. The mechanism of cell death in H1°-FokI transfected cells proceeds via an apoptotic pathway. The H1°-FokI transfected cells exhibit pronounced surface labeling with annexin V alexa fluor 488 and PI dye staining reveals extensively condensed nuclear DNA characteristic of apoptotic cell death. Scale bar = 10 μm.
A quantitative assessment of the percentage of cells in early apoptosis and late apoptosis or necrosis made at 72 hours post transient transfection was done by flow cytometry analysis of the annexin V alexa fluor 488 and propidium iodide stained cells (Figure 9, materials and methods). The percentage of cells in early apoptosis is high for live untransfected cells and all control transfections. Necrotic control cells show virtually no cells in early apoptosis. Camptothecin induced apoptotic controls show few cells in early apoptosis, but more in what is presumably late apoptosis. H1°-FokI transfected cells show more cells in early apoptosis than camptothecin treated cells but fewer than seen in live and control cells.

Following the transition of apoptosis and cell death through time (between 24-72 hours post-transfection, at 24 hours interval) by FACS analysis (Figure 10) shows that all populations, including the apoptotic-positive control cells, go into early apoptosis prior to the 72 hour time point except for the necrotic controls. During the first 48 hours post-transfection, relatively more H1°-FokI transfected cells were undergoing early stages of apoptosis than were in late apoptosis. However, there was a transition such that the majority of the H1°-FokI transfected cells experienced late stages of apoptosis 72 hours post-transfection. H1°-FokI transfected cells appear to progress from early (24-48 hours) to late (72 hours) apoptosis with no signs of necrotic death.

IV. Discussion

We have demonstrated that an H1°-FokI chimeric protein expressing gene can quite effectively kill cells into which it is transiently transfected. We created this gene, in part, to begin to establish the clinical potential of H1 to direct toxic agents, such as nucleases, to the nucleus in order to kill cells. Other toxic proteins have been created for this purpose, such as the caspases, to induce death by apoptosis (Yeh and Yen, 2005; Yakovlev and Faden, 2001; Moffatt et al, 2000). Because all linker histones localize predominately to the nucleus and bind to chromatin tightly (Mamoon et al, 2002) and abundantly (Widom, 1998; Simpson, 1978; Kornberg, 1974), we hypothesized that it could be used to deliver a sufficient amount of a toxic protein, such as a nuclease to efficiently kill cells. We know that the H1 histones

![Figure 9](image-url)  
**Figure. 9.** Transfection of mouse cells with H1°-FokI DNA causes cell death via a late apoptotic event. Mouse cells were transfected with various DNA constructs. Cells were harvested 72 hours PT and stained with the cell viability indicator annexin V alexa flour 488 and PI dyes. They were analyzed by flow cytometry procedure for determination of cell viability. Transfection of cells with the H1°-FokI DNA causes a decrease in cell viability in 63% of the cell population.
Figure. 10. Time-course analysis of the mechanism of cell death in H1°-FokI transfected cells. Mouse cells were transfected with various DNA constructs. Cells were harvested between 24-72 hours post-transfection, at intervals of 24 hours post-transfection and stained with the cell viability indicator annexin V alexa flour 488 and propidium iodide dyes. They were analyzed by flow cytometry procedure for determination of cell viability. Transfection of cells with the H1°-FokI DNA caused a decrease in cell viability in a time-dependent manner as compared to the control cells.

can carry the relatively large green fluorescent protein linked to their carboxyl terminal tail into the nucleus and bind to chromatin appropriately (Lever et al, 2000 and Misteli et al, 2000). We thought that it was also likely that the carboxyl tails, which lie along the linker DNA (Zhou et al., 1998; Pruss and Wolff, 1993), would position the nuclease such that it could readily cut the linker DNA. The in vivo turnover of H1 binding (Lever et al., 2000; Misteli et al., 2000) is also such that it would continue to come off and rebind to chromatin to further enhance the cleavage of the nuclear DNA. It seemed likely that such a chimera could cleave a sufficient amount of DNA such that the cellular repair systems are overwhelmed and the cells would die even if they had become resistant to apoptotic triggers (Sellers and Fischer, 1999; Gatti and Zunino, 2005). Although we demonstrate that H1°-FokI probably induces apoptosis, we have no reason to believe that the chimeric protein cannot still kill cells that have become resistant to apoptotic triggers; presumably it would eventually kill all cells through persistent cleavage of the nuclear DNA.

Because the experiments reported here were intended to be strictly a proof of principle, to see if H1 could be used to carry a nuclease into the cell and sufficiently fragment the nuclear DNA such that cell death would ensue, we chose transient transfection as the way to deliver the gene expressing the H1-nuclease chimera. Transient transfection is typically inefficient and its efficiency varies greatly, depending on cell type, vector and transfection protocol (Sambrook et al., 1989). We observed approximately 70% transfection efficiency and therefore did not expect to get 100% killing of the cells upon transient transfection with the H1°-FokI expression construct. Also, not all of the cells that become transfected will necessarily express sufficient amounts of H1°-FokI to result in cell death. We nevertheless, saw a killing efficiency of 63% (Figure 5) for the cells transfected with the H1°-FokI expressing construct. With methods of gene
delivery that result in higher copy numbers of exogenous genes, H1º-FokI will likely yield a 100% kill rate.

Although our data indicate that H1º-FokI transfected cells kill via an apoptotic mechanism, this does not mean that it would not kill cells that are resistant to apoptotic triggers. Except for the necrotic control cells generated by treatment with methanol, all of the cells that died probably did so by an apoptotic mechanism. It is known that transient transfection with plasmids in itself can kill cells and that this death in many cell lines is apoptotic (Rodriguez and Flemington, 1999).

H1 histones themselves have recently been shown to be potentially lethal to cells, (Tsoneva et al, 2005). In this study, the H1 was abundantly delivered by electroporation and the mechanism of killing was believed due to effects on the mitochondria. When delivered via a gene expression system, we observe no lethality due to H1 alone. This is in agreement with previous studies that relied on overexpression of H1 histones (Brown et al, 1996, 1997; Gunjan et al, 1990; Gunjan and Brown, 1999). We cannot exclude the possibility that the effect of H1 alone on viability is cell-type specific; electroloaded H1 did not have the same killing effect on non-transformed cells as it did on transformed cells (Tsoneva et al, 2005). However, we have not seen a lethality that can be attributed to H1 alone (Brown et al, 1996, 1997; Gunjan et al, 1990; Gunjan and Brown, 1999). However, we typically select permanent transfectants and the selection process may eliminate or select for resistance to H1 toxicity. We also cannot eliminate the possibility that some H1 variants are lethal. We have been unsuccessful in the selection of some permanent transfomers of H1 variants or mutants that can be induced to overproduce significant amounts of the particular H1 type (unpublished data).

It has been recently demonstrated that upon induction of apoptosis with agents that generate double-stranded breaks in DNA, such as with X-rays or with etoposide, the histone variant H1.2 (H1c) may be the apoptotic inducer (Konishi et al, 2003). As with the electroloaded H1s (Tsoneva et al, 2005), it appears to elicit a response through an interaction with mitochondria causing the release of cytochrome C. Overall, it appears that transfection with an H1 chimeric nuclease is a much more controllable method of killing cells; in situ cell specificity can be obtained by incorporating the H1-FokI gene into a viral vector that has been engineered to be cell type selective.

Acknowledgments
We thank Dr Susan Wellman for critical reading of the manuscript. We also thank Dr. Robert Lewis, Kevin Beason and Susan Touchstone for Fluorescent Activated Cell Sorter analysis.

References


Mamoon N, Song Y and Wellman SE (2002) Histone H1(0) and its carboxy-terminal domain bind in the major groove of DNA. Biochem 41, 9222-9228.


Alam and Sitman: H1º-FokI chimeric nuclease is cytoxic
Renz M (1975) Preferential and cooperative binding of histone H1 to mammalian chromosomal DNA. Proc Natl Acad Sci 72, 733-736.