Intracellular Combinatorial Chemistry with Peptides in Selection of Caspase-like Inhibitors

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Abstract

Fas is a cell surface receptor that can transmit signals for programmed cell death. Using a retroviral expression system, we have demonstrated that a short peptide derived from the cleavage site in a cellular target of a pro-apoptotic cysteine protease can be expressed within intact cells with sufficient activity to inhibit Fas-mediated apoptosis. *In vitro* analysis demonstrates that this retrovirally-expressed peptide is as potent as 150uM levels of the chemically synthesized peptide. Furthermore, using retroviral peptide library-based functional cloning we identified variants of this peptide with apparent anti-apoptotic activity. This approach is likely to lead to the identification of peptide variants with activity against a variety of signaling processes, both normal and pathological.

Introduction

Apoptosis is an important homeostatic mechanism that maintains cell number, positioning and differentiation. One of the best characterized processes for regulating apoptosis is initiated by the cell surface receptor Fas. Clustering of the Fas cytoplasmic domain by binding of Fas ligand or crosslinking antibody to the extracellular domain is capable of activating the interleukin-1b converting enzyme family of cysteine proteases (Caspases) - the proteolytic executioners of apoptosis (Enari et al., 1995)(Enari et al., 1996)(Los et al., 1995)(Tewari and Dixit, 1995). Recent studies implicate Caspase-8 (MACH/FLICE/Mch5) as a link between the Fas receptor and downstream Caspases via its association with FADD/MORT1, which itself associates with the Fas death domain (Boldin et al., 1996)(Fernandes-Alnemri et al., 1996)(Muzio et al., 1996). Other Caspases activated during Fas-mediated apoptosis include Caspase 1 (ICE) and Caspase 3 (Cpp32/Yama/Apopain)(Enari et al., 1996). The cleavage of a number of cellular substrates of the Caspases, including structural proteins, signaling molecules and DNA repair enzymes, coincides with the onset of apoptosis (Nicholson et al., 1995)(Na et al., 1996)(Tewari et al., 1995)(McConnell et al., 1997)(Zhivotovsky et al., 1997).

One of these substrates, Poly (ADP-ribose) polymerase (PARP), is proteolytically cleaved at the onset of apoptosis by Cpp32 (Caspase 3/Yama/Apopain)(Nicholson et al., 1995). The tetrapeptide aldehyde Ac-DEVD-CHO, corresponding to the cleavage site in PARP, residues 213-216, was shown to inhibit both the activity of CPP32 *in vitro* and the ability of apoptotic cell extracts to cause DNA fragmentation of isolated nuclei.

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(Nicholson et al., 1995)(Enari et al., 1996). The tetrapeptide aldehyde Ac-YVAD-CHO, corresponding to the cleavage site in IL-1b, a substrate for Caspase 1/ICE, has a similar, albeit weaker, anti-apoptotic effect (Thornberry et al., 1992)(Thornberry et al., 1994)(Enari et al., 1996). A difficulty in using such chemically synthesized peptides to modify apoptosis in intact cells is their poor entry into cells, short half-life and requirement for N- and C-terminal modification for effect (Thornberry, 1994).

We have devised a system for introducing short peptides into, and expressing within, living cells using retroviruses. The approach results in continuous expression of sufficiently high levels of peptide to modify intracellular signaling cascades. Here we demonstrate that a retrovirally-expressed, ribosomally-synthesized peptide corresponding to the Cpp32 cleavage site in PARP can, without any chemical modifications, protect cells from apoptosis mediated by Fas. Furthermore, we have selected variants of this peptide from a large, randomized library using a genetic screen within living mammalian cells. The results demonstrate the power of retroviral expression of short peptides for altering cellular phenotypes. Furthermore, the ability to select peptides of novel sequence with activity against apoptosis provides a new tool for isolating novel signaling proteins and for developing pharmaceutical leads capable of modulating pathological signaling processes.

Materials and Methods

Cell Lines: Phoenix-E producer cells expressing retroviral Gag, Pol and ecotropic envelope proteins were grown, and transfections performed, as per Pear et al (Pear et al., 1993). Briefly, cells were passaged into 6 cm tissue culture plates at 1.5 million per plate 18 hours prior to transfection. Transfections of vector or library DNA were accomplished by calcium phosphate precipitation. Cell media was adjusted to 25 uM chloroquine just prior to transfection. After application of precipitate, fresh media was added to the cells at 8 and again at 24 hours after transfection, at which time cells were transferred to 32°C for an additional 48 hours. Jurkat T cells expressing the mouse ecotropic retroviral (Baker et al., 1992) were resuspended at 5×10^5 cells per ml viral supernatant and spin infections were carried out in 24 well plates at 2500 rpm for 1.5 hours at 32°C, in the presence of 5uM polybrene. The plates were kept at 32°C for 10-14 hours, at which time cells were resuspended in fresh RPMI/10% FCS and transferred to 37°C for an additional 48 hours. Transduction efficiencies were estimated by independent transfections with ß-galactosidase retroviral vectors and FACS-Gal analysis of transduced cells (Nolan et al., 1988).

Vectors and Peptide Libraries: Synthetic oligonucleotide inserts encoding Caspase inhibitor peptides or peptide libraries were prepared as described previously (Mattheakis et al., 1994). Briefly, peptide or library oligonucleotides prepared by standard degenerate oligonucleotide synthesis were converted to double-stranded DNA inserts by primed DNA polymerization, digested with Bst XI and subcloned into the retroviral vector pMSCV/PC. Ligations were transformed by electroporation into *E. Coli* and the DNA recovered after overnight plating. For library preparation, a small aliquot of the combined electroporated samples was plated to determine library size. The remaining sample was inoculated directly into LB broth, incubated overnight and the library DNA prepared by Maxiprep (Qiagen). Insert sequence of the randomized region was NNKNNKNNKGAKNNK, where N encodes A,C,G or T and K encodes G or T.

Anti-Fas Selection: Jurkat T cells transduced with peptide or library vectors were selected with IgM anti-Fas antibody Ch-11 (Kamiya Corp.). Briefly, cells were resuspended in RPMI/2.5% FCS plus 50ng/ml CH-11 and distributed into 24 well plates

at 10^5 cells per well. After 5 days at 37°C, 1 ml RPMI/20% FCS was added to each well and the cells were placed at 37°C for an additional two - three weeks. Wells containing live cells were identified by visual inspection for nutrient depletion of the media and the presence of cell colonies. XTT assay of triplicate samples of each well were performed to confirm outgrowth of resistant cells.

Cpp 32 Enzyme Assays: Cell extracts were prepared and enzyme assays were carried out as per Lazebnik et al. (Lazebnik et al., 1993) Briefly, apoptotic extracts cells were prepared by repeated freeze/thawing and then were incubated with the chromogenic CPP32 substrate DEVD-pNA or the fluorogenic substrate DEVD-AMC (Clontech) in the presence or absence of increasing concentrations of chemically-synthesized CINP peptide. Substrate cleavage was measured in a plate reader or spectrofluorimeter for release of free chromogen/fluorophore.

LC/MS Analysis: After incubation of Fas-activated cellular extracts with chemically-synthesized CINP peptide, the extracts were passed over an HPLC column, desalted and loaded directly into a mass spectrometer. Individual major peaks in the size ranges expected for the full length peptide and potential cleavage products were scanned for peaks corresponding to the expected molecular weight values.

PCR Rescue and Library Analysis: Poly-A purified mRNA was prepared from surviving cells and RT-PCR carried out using the Titan RT-PCR kit (Boehringer-Mannheim) and primers flanking the peptide-encoding insert. Primers used were 5'GATCCTCCCTTTATCCAG3' and 5'CAGGTGGGGTCTTTCATTCC3'. Amplified samples were purified, digested with Bst XI and subcloned into the pMSCV/PC retroviral vector as described for peptide library generation. DNA was prepared from individual colonies and the sequence of each insert determined by cycle sequencing with the same upstream primer used for PCR rescue.

Statistical Analysis and Alignment of Sequences: Library sequences before and after selection were analyzed to determine the statistical significance of amino acid frequency differences. Separate comparisons for the completely randomized positions and the D/E position were carried out. The null hypothesis for each test was that there was no bias, so the observed frequency to the expected frequency of each amino acid given the nucleotide degeneracy was compared. The significance of the deviation was noted at the highest level of confidence given the results of a G-test (Microsoft Excel).

Results

Expression of Caspase inhibitor peptides can rescue T cells from Fas-mediated apoptosis in long-term assays.

To determine whether a peptide could inhibit apoptosis when expressed in cells with retroviruses, the retroviral vector pMSCV/PC/CINP was constructed (Figure 1). This construct encodes residues 209-218 of PARP, including the scissile bond D216-G217 downstream of a Kozak translation consensus sequence (Kozak, 1986) and should result in translation of the free peptide, without any N- or C-terminal chemically synthetic modifications, when expressed in cells. Jurkat T cells were transduced with virus prepared with this construct, a b-galactosidase (b-gal) construct or several nonspecific peptide constructs. Each transduced cell population was divided into 10 wells of a 24 well plate

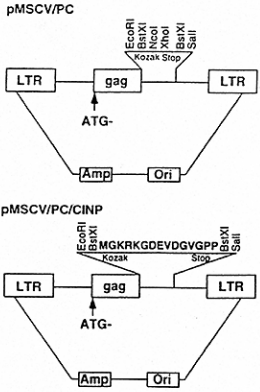

Figure 1
Schematic structures of pMSCV/PC and pMSCV/PC/CINP vectors. The pMSCV/PC vector (*Upper)* encodes a Kozak translation initiation site, stop codon and peptide stabilizing residues within two nonidentical BstXI sites. These sites were used to create the pBabe/PC/CINP vector (*Lower)*, which encodes the potential Caspase inhibitor peptide.

at 10^5 cells per well and stimulated with CH-11, a monoclonal antibody against the Fas receptor. After two weeks, cell survival in each well was assayed in triplicate using XTT (Roehm et al., 1991). As shown in Figure 2, the two Jurkat T cell populations independently transduced with the pMSCV/PC/CINP construct showed significantly increased survival by XTT assay compared to T cells transduced with b-gal or any of the nonspecific peptide constructs.

Mechanism of peptide action: pseudosubstrate inhibition.

The DEVD peptide encoded by the pMSCV/PC/CINP construct was able to protect cells transduced with it from Fas-induced apoptosis, even though it contains the scissile D216-G217 bond in PARP actually cleaved by CPP32 and lacks the C-terminal aldehyde required for covalent inactivation of the enzyme's catalytic cysteine (Thornberry et al., 1994). To gain some insight into the expressed peptide's mechanism of protection, we compared residual Cpp32 enzyme activity against the added chromogenic substrate DEVD-AMC in apoptotic cell extracts which had been mixed with the chemically synthesized CINP peptide to the activity in extracts of apoptotic cells that had been transduced with the retroviral vector encoding the same peptide. The amount of residual CPP 32 enzyme activity in transduced cells was equivalent to the residual activity in untransduced extracts containing 150uM chemically synthesized peptide (data not shown). We conclude that a PARP cleavage site peptide without any chemical modifications can serve as a pseudosubstrate inhibitor of Cpp32, decreasing the amount of endogenous substrate cleavage enough to protect cells from apoptosis if present at sufficiently high

concentration, even if the peptide itself is cleaved. However, we doubt that the actual concentration of the peptide in cells approaches 150uM. We are currently exploring the possibility that the peptide acts in some other manner (see discussion) to give this apparent inhibitory concentration.

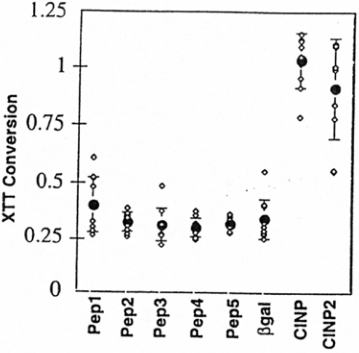

Figure 2
Retrovirally-expressed CINP peptide protects cells from Fas-induced apoptosis. Jurkat T cells were transduced with peptide control virus (Pep 1-5), b-gal virus or virus encoding the CINP peptide (CINP and CINP2). Cells were distributed into 24-well plates and treated with anti-Fas monoclonal CH-11 as described in Methods. Cell outgrowth after antibody treatment was monitored by XTT assay. Data shown are the results of 10 independent cell samples, with XTT measurement for each data point done in duplicate.

To determine whether the CINP peptide itself was capable of being cleaved by activated cell extracts, the chemically synthesized CINP peptide was mixed with Fas-activated apoptotic cell extracts, and LC/MS analysis carried out on the cleavage products. As shown in Figure 3, prominent peaks corresponding to the molecular weights of the products expected from cleavage by Cpp32 were found (peaks 2 and 4a), as well as two peaks corresponding to products formed by the cleavage of peptide bonds not recognized by cysteine proteases (peaks 3 and 4b). The presence of such additional cleavage products is not surprising, given that extracts of cells could contain a variety of endo- and exopeptidases in addition to cysteine proteases. These results suggest that the CINP peptide is capable of being cleaved by activated Cpp32 and support the hypothesis that overexpression of a peptide substrate of Cpp32 can decreases the amount of endogenous substrate cleavage sufficiently to protect cells in which Cpp32 has been activated from apoptosis.

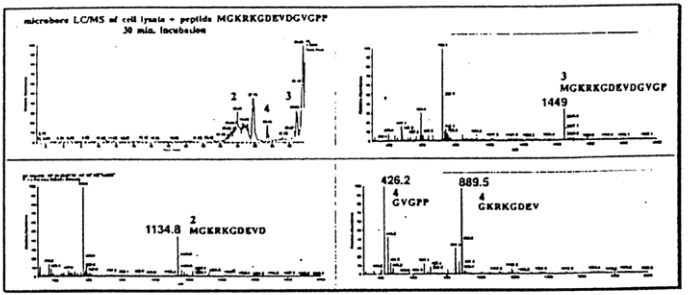

Figure 3
Mass spectrometry analysis of peptide activity in Fas-activated cellular extracts. Peptide was incubated in cellular extract from activated activated Jurkat T cells. Extracts were passed over HPLC, desalted, and run directly into the mass spectrometer. Individual major peaks in the range of sizes expected for the peptide and its cleavage products were scanned for peaks corresponding to expected value. Major peaks were found for the products in peaks 2 and 4. Peak 4 also contained a peptide product corresponding to an unknown proteolytic mechanism (GKRKGDEV).

Selection of peptides within T cells shows statistical variance from randomness.

To verify that rare retrovirus expression constructs could be phenotypically selected from a large library using the Phoenix system, co-transfections were carried out with a b-gal retrovirus vector and serial molar dilutions of a neomycin-resistance *(neo)* retroviral vector (Figure 4). After transduction of NIH 3T3 cells and selection in 1 mg/ml G418, neomycin-resistant colonies were detected down to 1 neo construct in 10^7 β-gal constructs. In such

βgal	1	1	1	1	1	1	1
Neo	1->10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	0
Colonies	Confluent	60	20	31	10	0	0

Figure 4
The Phoenix retroviral packaging system can select for rare phenotypes. Phoenix producer cells were co-transfected with a b-galactosidase retroviral construct (bgal) and serial molar dilutions of a neomycin resistance gene-encoding retroviral construct (Neo). 48 hours later, the viral supernatant was used to transduce populations of NIH 3T3 cells, which were selected in 1mg/ml G418. The number of G418-resistant cell colonies after two weeks of selection is shown.

experiments, the b-gal constructs represent the total library and the *neo* constructs the rare members of the library capable of conferring a selectable phenotype on the target cells. Thus, the Phoenix library system can detect rare events against a high background of undesirable events, with a sufficiently strong selection.

We next determined whether the Caspase inhibitor peptide described above or variants of it could be identified by a genetic selection in mammalian cells for peptide inhibitors of Fas-mediated apoptosis. A retroviral construct library was generated in which the nucleotide positions corresponding to amino acids P4,P3,P2 and P-1 were completely randomized, while the degeneracy in nucleotides corresponding to position P1 was limited to permit the generation of only Aspartate or Glutamate at that position in the library (Figure 5). It is known that an Aspartate at the P1 position is strongly preferred for substrate cleavage by all known Caspases, while position P4 is important for substrate recognition, with ICE-like proteases having a preference for hydrophobic residues like Tyrosine at P4 and CED-3/CPP32-like proteases having a preference for Aspartate (Rano et al., 1997)(Rano et al., 1997). Among known endogenous substrates for Caspases, positions P2 and P3 show the most variability. An analysis of the sequence of 25 clones from the unselected DNA

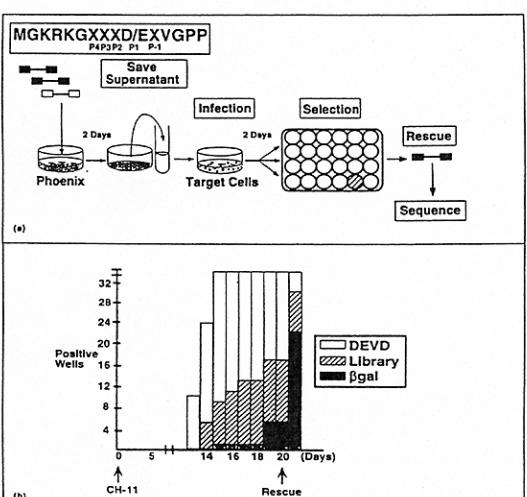

Figure 5
Genetic selection for variants of CINP that inhibit Fas-induced apoptosis. (a) Jurkat T cells transduced with peptide library virus (see Materials and Methods) were distributed into 24 well plates at 100,000 cells per well and treated with 50 ng/ml CH-11 monoclonal IgM antibody against the Fas receptor. (b) Wells containing live cells were identified by visual inspection and XTT assay.

construct library demonstrated no statistically significant variation from expected randomness (data not shown). The library was transfected into Phoenix Eco and Jurkat T cells cells expressing the ecotropic viral receptor (Baker et al., 1992) were transduced with the resulting virus. 10^7 cells were divided into 100 wells of a 24 well plate and selected in the presence of CH-11 antibody as described above (Figure 5a). After two weeks, wells containing surviving cells were examined by visual inspection for nutrient depletion in the media and the presence of cell colonies. There was a significant increase in positive wells of cells transduced with the library, as compared to cells transduced with a b-gal vector (17 for the library versus 6 for b-gal by day 22; Figure 5b). As expected, each well of cells that had been transduced with the pMSCV/PC/CINP construct contained live cells.

To identify the peptides that might be expressed in the surviving cells, RT-PCR was carried out on cells from each well (data not shown). After PCR amplification each library sample contained a predominant PCR band of the expected size, plus nonspecific bands; restriction

(a)

Amino Acid	potential codons	Observed no selection	Observed selection	Expected if Random
non-P1				
F	1	4	5	3.1
L	3	14	8	9.4
S	3	7	9	9.4
Y	1	3	7	3.1
C	1	4	0	3.1
W	1	2	1	3.1
P	2	9	5	6.3
H	1	3	0	3.1
Q	1	2	2	3.1
R	3	12	8	9.4
I	1	3	4	3.1
M	1	4	7	3.1
T	2	4	13	6.3
N	1	5	5	3.1
K	1	2	1	3.1
A	2	8	11	6.3
D	1	3	5	3.1
E	1	1	3	3.1
G	2	2	2	6.3
V	2	4	13	6.3
STP	1	4	0	3.1
P1				
D	1	12	21	12.5
E	1	13	5	12.5

Figure 6

(a) Table of amino acid frequencies for selected peptides. Sequences were determined for individual unselected and *in vivo* selected peptides, and the frequencies of individual residues compared in the two groups. Table shows observed amino acid frequencies before and after selection as well as the frequencies expected from a purely random library. (b) Clustal W aligned peptides selected after Fas treatment. Sequences are presented as grouped by the PAM 250 weight matrix into most similar sequences.

(b)

TASDA		SDRDI
RASDL		VLGDR
TPSDM		LSYDR
YPSDV		SYQDL
YASDV		MTPDP
YTSDA		MTIEA
YAVDE		WLLEF
YARDI		
RKTDA		RNTET
QTTDA		RETER
IVNDT		VVDEM
FGNDF		VVNEM
FPDDL		
FADDL		

digest with Bst XI generated the expected peptide-encoding insert plus the digested end products. Interestingly, none of 6 cell samples from the b-gal-transduced cells generated an RT-PCR product. Since the initial transduction efficiency was approximately 15% as measured by X-Gal of transduced cells, and since b- gal should not confer any survival advantage in the presence of Fas stimulation, the frequency of surviving cells actually transduced with a b-gal virus should approximate the initial transduction efficiency (15%). Therefore, the apparent lack of b-gal-transduced surviving cells among the negative control samples is not surprising.

For 10 of the 17 samples surviving from the library, the RT-PCR product was ligated into pMSCV/PC, electroporated into *E. Coli* and plated on LB/Amp plates overnight. The DNA prepared from 10 colonies for each sample was sequenced to identify the encoded peptide(s). An analysis of the types of residues in the selected versus the unselected library is shown in Figure 6a. A highly significant increase in the frequency of Asp in position P1 (21/26, or 83%, $p < 0.001$) was observed versus the frequency in the unselected library (50%), implying that cells expressing peptides with Asp in this position possess a survival advantage in the presence of Fas stimulation. Furthermore, the observed amino acid frequencies at the other positions differed significantly from the random distribution of residues expected if no selection had occurred.

A Pam 250 Weight Matrix of the recovered peptide sequences is shown in Figure 6b. Interestingly, several of the peptides recovered by the screen appear similar in sequence to actual or predicted cleavage sites in endogenous substrates of the Caspases (see discussion).

Discussion

The ability of peptides or peptide-like compounds to alter the physiology of cells is well documented from the examples of peptide-like antibiotics, anticancer agents and immune modulators (Moreno et al., 1995)(Katz and Donadio, 1995)(Gasson, 1995). However, it has never been reproducible demonstrated in a stably-expressing system that such peptides can confer significant long-term consequences. Furthermore, the full activity of such compounds often derives from post-translational modification of a ribosomally-synthesized pro-peptide form of the compound, for example cyclization or amino- or carboxy-terminal modification. Here, we demonstrate that a ribosomally synthesized, unmodified linear peptide can potently alter the physiology of mammalian cells when expressed in such cells with retroviruses. The retrovirally expressed CINP peptide, derived from the primary amino acid sequence of PARP, a cellular substrate of the prop-apoptotic cysteine protease Cpp32, is capable of protecting those cells from apoptosis induced by Fas (Figure 2). Consistent with this protective effect is the demonstration that apoptotic extracts of cells expressing this peptide contain signficantly reduced Cpp32 enzyme activity toward a chromogenic peptide substrate (data not shown).

Comparison of the residual Cpp32 activity in the extracts of apoptotic cells overexpressing the CINP peptide to extracts mixed with the chemically synthesized CINP peptide suggests that retroviral expression of the peptide can decrease Cpp32 activity in the extracts to the level of 150uM of chemically synthesized peptide added to such extracts (data not shown). Because cell contents are diluted during preparation of the extracts, the actual intracellular concentration would be predicted to be significantly higher. With retroviral expression, however, the peptide is present in the cell before pro-Cpp32 is cleaved to its activated form through induction by an apoptotic stimulus. Because this activation depends on cleavage of pro-Cpp32 at two Asp-Gly bonds contained within cleavage sites that fit the consensus for Cpp32 substrates, it is thought that Cpp32 might act on itself in a self-amplification reaction, or that the proenzyme is cleaved by other upstream cysteine proteases, for example ICE (Enari et al., 1996)(Faleiro et al., 1997)(Takahashi et al., 1997). Therefore, the reduction in Cpp32 enzyme activity observed in extracts of cells overexpressing the CINP peptide through retroviral transduction could be due to a decrease in the total amount of pro-Cpp32 cleaved to active Cpp32, in addition to direct inhibition of the activated enzyme. The activation state of Cpp32 could be determined by Western blot of cell extracts using antibodies that recognize both the pro- and activated form of the enzyme: decreased activation would be reflected in an increase in the higher molecular weight uncleaved pro form (and a decrease in the lower molecular weight cleavage products). Although we could detect cleaved PARP (a Cpp32 substrate) in extracts of Fas antibody-treated cells by Western blot with anti-PARP antibodies, we were unable to detect cleaved Cpp32 in the same extracts with a monoclonal antibody to the p12 subunit of Cpp32, which is present in both the pro- and activated forms of the enzyme (Y.H., unpublished results). A possible explanation is that too little pro-Cpp32 is cleaved to the activated enzyme to be detected by Western blot. A second approach to detecting cleaved Cpp32 is to mix the purified, ^{35}S-methionine-labelled enzyme with apoptotic cell extracts and then detect the cleaved products by PAGE. However, while this might permit us to determine whether retrovirally-expressed CINP peptide is capable of decreasing the cleavage of labeled proCpp32 by endogenous Cpp32 in the activated extracts, it does not permit us to determine whether the peptide can decrease the initial activation of Cpp32 in intact cells. We are currently attempting to label the activated Caspases in cell extracts with a biotinylated aldehyde inhibitor biotinDEVD-CHO, which might permit purification and qunatitation of the amounts of activated endogenous Caspases in cellular extracts using Streptavidin-conjugated secondary reagents (Faleiro et al., 1997).

The ability of the fifteen amino acid ribosomally-synthesized CINP peptide to protect cells from apoptosis suggested the possibility of using our retroviral peptide expression technology in a genetic selection for CINP variants which protect cells from apoptosis (Figure 5a). A retroviral construct library was designed in which selected residues in the CINP sequence were completely randomized while the key Aspartate residue at the P1 position was either maintained or mutated to Glutamate. We have previously shown that a Glutamate at P1 renders the retrovirally-expressed peptide inactive (S.M.R., unpublished data). The library was introduced into Jurkat T cells by the retroviral technique, and the cells were treated with the CH-11 monoclonal antibody against Fas to induce apoptosis. We observed an enrichment in wells containing living cell colonies in samples transduced with the library versus those transduced with a b-gal vector (figure 5b). The highly significant bias towards Aspartate in position P1 of the recovered peptide sequences is expected if Asp at P1 is critical to the activity of the peptide in protecting the cells from apoptosis. A comparison of the recovered peptide sequences to known or predicted cysteine protease cleavage sites shows a number of similarities (Figure 6). Of particular interest is a family of related sequences (YASDV,YTSDA,YAVDE,YARDI) which are quite similar to the cleavage site in interleukin-1b (YVADA). It has previously been shown that the tetrapeptide aldehyde YVAD-CHO is capable of inhibiting apoptosis in Jurkat T cells induced by Fas (Enari et al., 1995). Therefore, it is not surprising that our screen might have selected peptides with activity against other with required activity in inducing apoptosis. We are currently in the process of introducing the recovered peptides individually into cells to determine which can protect cells from Fas-induced apoptosis. We are also testing each peptide for its specific ability to inhibit the activity of the several known Caspases.

Not present in the recovered peptide sequences is a sequence encoding the CINP peptide itself, which should have been present in the original construct library at a frequency of 1 in 500,000. A possible explanation is that during library virus production a CINP-encoding sequence was not present. Ten million Jurkat T cells were actually used for transduction, which at a 30% transduction efficiently should have produced approximately three million transduced cells, or 1.5 times the theoretical total library construct size of two million at the nucleotide level. When considered with the wide heterogeneity in expression achieved with retroviral infection, it is therefore not unexpected that the CINP peptide sequence was not recovered from the surviving cells, either because it was never introduced into the cells before selection, or because it was introduced, but expressed at too low of a level to permit the transduced cell(s) to survive the anti-Fas selection. Another explanation is that numerous peptides in the library are capable of inhibiting apoptosis by acting at other points in the cascade or by more efficiently inhibiting other important Caspases. Since our peptides are conformationally flexible, it is possible that certain linear motifs assume more rigid structures capable of inhibiting distinct Caspases more efficiently. These and other possibilities will be tested.

Importantly, the technique described here offers the possibility that protein-protein interactions, often defined by linear peptide interfaces, might be disrupted or co-opted through expression of short peptides. Thus, a panoply of potential new gene therapeutics, derived from known protein interfaces, could be developed and delivered by viral or other expression techniques. The system has the advantage that short peptides might be largely non-immunogenic. As well, crucial determinants from proteins might be isolated for therapeutic effect while not introducing other protein determinants that provide unnecessary or pathological side effects.

We have demonstrated that a ribosomally synthesized linear peptide derived from a substrate for the pro-apoptotic cysteine protease Cpp32 can inhibit Fas-mediated apoptosis in Jurkat T cells when expressed in those cells with retroviruses. Furthermore, we have demonstrated that variants of this peptide can be selected from a large library. Our approach is broadly applicable to any physiological process which can be modeled in cell culture. We are currently in the process of screening retroviral peptide libraries for peptides with activity against signaling pathways involved in cancer, immune cell dysfunction and viral infection. Such peptides would represent ligands for isolating the proteins involved in these processes as well as potential lead compounds for pharmaceutical development.

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