Inhibitors of Interleukin-4 Signaling in Human B Cells*

Retrovirally Delivered Random Cyclic Peptide Libraries Yield

Some of the most common scaffolds utilized in nature for producing high-affinity drug-like effectors are based on cyclized peptide architectures. Both naturally occurring and synthetically designed cyclic peptides have been successfully employed as drugs in man (1–5). These cyclic peptides often exhibit enhanced binding to macromolecules due to a restricted conformation space, and lowered conformational entropy loss upon binding and diminished proteolytic susceptibility (3, 4). By utilizing the protein splicing and ligation properties of inteins, it is now possible to direct synthesis of diversity-oriented, cyclic peptides in mammalian cells using retroviral technology. These results demonstrate the generation of cyclic peptide libraries in human cells and the power of functional screening to rapidly identify biologically active peptides.

Inteins are polypeptide sequences found in a small set of primarily bacterial proteins that promote the splicing of flanking pre-protein sequences to generate mature protein products. Inteins can be engineered in a “split and inverted” configuration such that the protein splicing product is a cyclic polypeptide consisting of the sequence linking two intein subdomains. We have engineered a split intein into a retroviral expression system to enable the intracellular delivery of a library of random cyclic peptides in human cells. Cyclization of peptides could be detected in cell lysates using mass spectrometry. A functional genetic screen to identify 5-amino acid-long cyclic peptides that block interleukin-4 mediated IgE class switching in B cells yielded 13 peptides that selectively inhibited germ line e transcripts. These results demonstrate the generation of cyclic peptide libraries in human cells and the power of functional screening to rapidly identify biologically active peptides.

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Todd M. Kinsella‡, Cara T. Ohashi‡, Amy Grace Harder, George C. Yam, Weiqin Li, Beau Pellele, Erlina S. Pali, Mark K. Bennett, Susan M. Molineaux, D. A. Anderson, Esteban S. Masuda, and Donald G. Payan†
From Rigel, Inc., South San Francisco, California 94080

‡ To whom correspondence should be addressed: Rigel, Inc., 240 East Grand Ave., South San Francisco, CA 94080. Tel.: 650-624-1102; Fax: 650-624-1133; E-mail: dgpayan@rigel.com.

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‡ These authors contributed equally to this work.
§ Present address: Dept. of Biology, Massachusetts Inst. of Technology, Cambridge, MA 02139-4307.
¶ To whom correspondence should be addressed: Rigel, Inc., 240 East Grand Ave., South San Francisco, CA 94080. Tel.: 650-624-1102; Fax: 650-624-1133; E-mail: dgpayan@rigel.com.

§ The abbreviations used are: IL-4, interleukin-4; GFP, green fluorescent protein; BFP, blue fluorescent protein; FACS, fluorescence-activated cell sorter; dox, doxycycline; RT, reverse transcriptase; TGF-β, transforming growth factor-β; MS, mass spectrometry; HA, hemagglutinin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-

flight; LTR, long terminal repeat; HBEGF, heparin-binding epidermal growth factor.
mants for this library was estimated at 2.7 × 10^5.

Functional Retroviral-based Screening—Production of infectious retroviral particles in PhoenixA cells and infection of target cells were carried as described in Swift et al. (11). Approximately, 1 × 10^4 Phoenix A packaging cell line cells were transfected with 20 μg of retroviral random cyclic peptide library plasmid DNA, and the resultant cyclic peptide library viral particles were used to infect A5T4 cells. Positive (PYGYDSGDAYS) and negative (LALGHQKEYHDTPPADKTTELGP) control linear peptides identified in a previous screen (data not shown) were included to monitor the screen selection process. Four days post-infection, the cells were stimulated with IL-4 (30 units/ml final concentration; Peprotech, Rocky Hill, NJ) for 24 h followed by addition of diphtheria toxin (20 ng/ml final concentration; Calbiochem) to kill cells expressing the HBEGF diphtheria toxin receptor. Forty-eight hours later, the cells were washed twice with fresh medium to remove excess diphtheria toxin. Cell death was monitored by propidium iodide staining of the cells and FACS analysis. Cells were passed over a Ficoll (Amersham Biosciences) gradient at 3 and 5 days post-diphtheria toxin washout to remove dead/dying cells from culture. The surviving cells were expanded in the presence of doxycycline (dox) to suppress library expression and then stimulated with IL-4 (30 units/ml). Cells retaining the ability to respond to IL-4 were identified based on induction of GFP and single cell cloned into 96-well plates on MoFlo (Cytomation, Ft. Collins, CO) cell sorters.

After single cell clones had expanded, cells were replica-plated and grown in the presence or absence of dox (100 ng/ml) for 4 days. Both plates were then stimulated with IL-4 and clonal GFP induction monitored by flow cytometry. Using geometric mean for GFP expression, the clones were ranked by calculating the ratio of dox (peptide off) geometric mean over dox (peptide on) geometric mean.

Rescue and Confirmation of Library Member Inhibitors—Once the potential inhibitors had been identified and ranked (cut-off ratio of 1.15), the random peptide inserts were rescued by PCR and ligated into the naive library vector. RT-PCR was performed using Titan RT-PCR kit (Roche Molecular Biochemicals) and 5′-CGTTTCTGATAGGCAC-CTATTGGTC-3′ as the forward primer and 5′-GATGCTCACCCTTCT-.
GCCGGTG-3' as the reverse primer. Cycle conditions were 50 °C for 30 min and 94 °C for 2 min. This was followed by 40 cycles, each consisting of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s and completed with a final extension of 10 min at 72 °C. The resulting PCR reaction was cleaned using a Qiaquick column (Qiagen, Valencia, CA). Sequential restriction enzyme cuts were made using MluI at 37 °C followed by BclI (both from New England Biolabs, Beverly, MA) at 50 °C. Digests were run on 2% agarose gels and purified using a Qiagen column. The screen is chronologically ordered by days. The principal steps are labeled and indicated by boxes. A5T4 cells infected with the retroviral intein S + 4 library were stimulated with 30 units/ml IL-4 to cause transcription of HBEGF regulated by a truncated fragment of the e promoter. Twenty-four hours post-stimulation, 20 ng/ml diphtheria toxin was added to induce killing and select for survivors. FACS profiles of cell populations stained with propidium iodide at days 7, 15, and 21 are shown in the inset above (gating indicates live cell population). Day 21 marks the recovery of those cells surviving diphtheria toxin selection. The expression of peptide library members was controlled by the presence (in red) or absence (in blue) of dox. With the peptide turned off, the survivors were stimulated with IL-4 and the IL-4 responders (GFP cells) were single cell-sorted. Single cell clones containing inhibitory peptides were selected based on GFP fluorescent inhibition in the absence of dox. A representative GFP FACS profile for a dox-regulatable clone is shown on the right.

**FIG. 2.** Functional genetic screen in BJAB cells for retrovirally expressed cyclic peptides blocking IL-4 induced transcription of the IgE e promoter. A, schematic representation of the selection system. The A5T4 screening cell line contains a dual function reporter, HBEGF-2a-GFP, driven by a 600-bp fragment from the e promoter (Pe). Upon stimulation with IL-4, HBEGF is ectopically expressed and sensitizes the cells to be killed by diphtheria toxin. The S + 4 library containing potential inhibitors is retrovirally infected into the screening cell line. Its expression is controlled by tetracycline-responsive elements (TRE), which respond to tTA-VP16 (tTA). The amino acid residues at the splicing junctions that are underlined were replaced with alanines to create mutant non-splicing inteins. X, any amino acid; LTR, LTR(U3) promoter; SIN, mutated non-functional LTR promoter; PA, polyadenylation site; IL-4R, interleukin 4 receptor. B, scheme for the functional screen of the S + 4 library. The screen is chronologically ordered by days. The principal steps are labeled and indicated by boxes. A5T4 cells infected with the retroviral intein S + 4 library were stimulated with 30 units/ml IL-4 to cause transcription of HBEGF regulated by a truncated fragment of the e promoter. Twenty-four hours post-stimulation, 20 ng/ml diphtheria toxin was added to induce killing and select for survivors. FACS profiles of cell populations stained with propidium iodide at days 7, 15, and 21 are shown in the inset above (gating indicates live cell population). Day 21 marks the recovery of those cells surviving diphtheria toxin selection. The expression of peptide library members was controlled by the presence (in red) or absence (in blue) of dox. With the peptide turned off, the survivors were stimulated with IL-4 and the IL-4 responders (GFP cells) were single cell-sorted. Single cell clones containing inhibitory peptides were selected based on GFP fluorescent inhibition in the absence of dox. A representative GFP FACS profile for a dox-regulatable clone is shown on the right.

**Measurement of Germ Line e Expression**—To study the effects of the active cyclic peptides on germ line e transcription, we used quantitative real time PCR (TaqMan, Applied Biosystems, Foster City, CA) to determine the levels of the e transcript. Cells were first sorted for BFP to ensure that they were retrovirally infected. Four different assay conditions were tested: −dox + IL-4, + dox − IL-4, + dox + IL-4, − dox − IL-4. All experiments were conducted in triplicate. BFP positive sorted cells were thereafter maintained in dox (peptide off). To obtain the −dox condition, cells were washed two times with medium and maintained in dox-free culture for over 72 h. Cells in ± dox were split to 50,000 cells.

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per ml. The following day, IL-4 was added (30 units/ml), and cells were incubated for 3 days before FACS analysis and pelleting for RNA extraction. The TaqMan primers were as follows: forward primer, 5'-ATCCACAGGCACCAAATGGA-3'; reverse primer, 5'-GGAAGACGAGATGGGCTCTG-3'; and the probe, 5'-(6-carboxyfluorescein)-ACCAGGCGCTTCAGCCTCCA-(6-carboxy-N,N,N'-tetramethylrhodamine)-3'. The probe spans a splice junction and thus will not detect contaminating DNA in the reaction assay. The One-step RT-PCR kit (Applied Biosystems) was used for the RT-PCR reaction. To normalize the results, the housekeeping gene GAPDH (Applied Biosystems) was used. This value was considered statistically significant if p < 0.05.

To assess specificity of the activity of the cyclic peptides for the ε promoter, peptides decreasing ε promoter activity were tested in the B cell line, ST486, for inhibition of germ line α promoter activity when cells were induced with TGF-β. The ST486 cell line used was engineered to express the tTA-VP16 ires hygromycin phosphotransferase gene. α transcript quantification was determined in the same manner as ε transcript quantitation. The primers used were as follows: forward primer, 5'-CAGACCTGGGCCC-3'; reverse primer, 5'-TCAGGCG-GAAAGACCTGG-3'; and probe 5'-(6-carboxyfluorescein)-CCAGACGCTTGACAGGATCC-3'. As with the ε primer/probe set, the alpha probe spans a splice junction and does not detect contaminating DNA. None of the peptides inhibited α transcription.

Detection of Cyclic Peptides by Mass Spectrometry—A5T4 BJAB cells (5 × 10^7) were lysed in 400 μl of PBS (MgCl2-free) containing 4% Tween 20, 5 mM EDTA, 2 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride, and a tablet of protease inhibitors (Roche Biochemicals) by the freeze-thaw method. Proteins were precipitated by the addition of trifluoroacetic acid to 0.1% v/v. The lysate was centrifuged 30 min at 14 K (4°C) to remove precipitated proteins, and the supernatant was passed through a 3-kDa cut-off filter (Millipore Corp., Bedford, MA) and concentrated by vacuum centrifugation to ~50 μl. This solution was injected onto a C18SP 5 cm x 1 mm inner diameter Vydac (Hesperia, CA) reversed phase column eluted on an HP1100 HPLC (Hewlett-Packard, Palo Alto, CA) at 100 μl/min, and 1-min fractions were collected. These were centered around the elution position of the synthetic standard peptide (from American Peptide Co., Sunnyvale, CA) when available. One microliter of each fraction was mixed with 1 μl of dihydroxybenzoate matrix solution (Agilent, Palo Alto, CA; diluted 10-fold with 50% v/v acetonitrile, 0.1% v/v trifluoroacetic acid). One microliter of this mixture was spotted onto a 400 micron well of an Anchor Chip (Bruker, Billerica, MA), and the mass spectrum was collected on a Bruker Reflex III time-of-flight mass spectrometer. Masses were assigned after external calibration using angiotensin III and the peptide MRFA; the mass accuracy was 100 ppm. MS/MS spectra were collected on an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) after injection of the peptide fraction onto a capillary C18 reversed phase column eluted into the mass spectrometer source.

RESULTS AND DISCUSSION

Intein-mediated Protein Splicing in Mammalian Cells—We first used retroviral vectors to express different versions of the...
DnaB intein from Synechocystis sp. (Ssp) PCC6803 in human BJAB lymphocytes to examine the splicing function of a prokaryotic intein in human cells (Fig. 1A). The inteins were inserted into the third external loop of GFP (9), which allowed close proximity for the intein splice junctions to interact (12). Intein splicing was monitored by detecting the relative molecular weight of GFP species by Western blotting (Fig. 1B) using α-GFP antibodies (constructs 1–4) or α-HA antibodies that recognized the influenza HA epitope tagged at the C termini of constructs 2 and 4 (Fig. 1B, α-GFP and α-HA panels). In addition, to monitor the splicing fidelity, Myc epitope half-sites were engineered into GFP loop 3 flanking the intein junctions (constructs 3 and 4), such that accurate protein splicing would reconstitute a full Myc epitope that could be recognized by α-Myc antibody (Fig. 1B, α-Myc panel). The results indicate that protein splicing, which removed the intein in constructs 1 and 2, was very efficient and appeared complete (Fig. 1B). Splicing for constructs 3 and 4, although less efficient, demonstrated accurate reconstitution of the Myc epitope. The drop in efficiency is likely due to a preference for particular residues near the splice junction (5, 13). These results demonstrate that intein-mediated splicing can proceed with efficiency and precision in mammalian cells.

**Generation of a Retrovirally Expressed Cyclic Peptide Library**—We next constructed a GFP-tagged retroviral vector, construct 5, driving the expression of the test peptide RGD7 (SRGDGWS) inserted between the two components of a split-intein in an inverted configuration to generate a cyclic peptide using the Ssp DnaB intein in BJAB cells (Fig. 1C). Such a configuration, Int C-peptide-Int N, has been previously shown to result in cyclization of peptides using the Ssp DnaE intein in E. coli (14, 15). To demonstrate cyclic peptide production in BJAB cells, we developed a purification method for small cyclic peptides using the synthetic cyclic RGD7 peptide spiked into cell lysates of naive cells (see “Experimental Procedures”). The method was then applied to lysates of BJAB cells that had been infected with construct 5 and FACS sorted for GFP fluorescence into a homogeneous population expressing the Int C-RGD7-Int N GFP fusion. A peptide with a mass and MS/MS fragmentation pattern nearly identical to the predicted or spiked synthetic cyclic peptide was observed by mass spectrometry (Fig. 1D). Thus, inverted Ssp DnaB split inteins expressed in human cells efficiently cyclized the RGD7 peptide in vivo.

We then replaced the sequences encoding SRGDGWS with sequences encoding the catalytically required serine residue followed by random 4-mer peptides (S+4 library) to create a retroviral expressed cyclic peptide library. The random peptides were encoded by the DNA bases NNK-NNK-NNK-NNK (where N = A, C, G, T and K = G, T). In addition, the intein sequences were optimized for human codon usage, which resulted in increased expression (data not shown). Furthermore, the intein-library peptide construct was fused to BFP to enable identification of the cells expressing the different library members. The library was constructed in the pTRA retroviral vector, which rendered viral insert expression tetracycline-dependent (10). The final configuration of the cyclic library in the context of the retroviral vector construct following insertion into the genome of infected cells is shown in Fig. 2A. The potential complexity of the library is about 160,000 members at the amino acid level and about 1 million members at the DNA codon usage level. The number of library members that actually proceed to cyclization is unknown but detailed analysis by Scott et al. (5) of an analogous library in E. coli cells indicated that about 70% of the library members could produce cyclic products and that there is modest amino acid distribution biases at the different random positions.

**FIG. 4. Effects of expression of peptide library members on IL-4-dependent endogenous ε germ line transcription in the context of both wild-type (A) and mutant intein (B).** Germline ε promoter transcription was measured by quantitative RT-PCR (Taqman, Applied Biosystems). The relative expression values (n = 3) indicated IL-4-induced ε transcript levels in cells infected with the designated library member in the presence of peptide (−dox, solid bars) or the absence of peptide (+dox, empty bars). Uninfected A5T4 cells were used as a negative control. A5T4 cells expressing SOCS-1, a known IL-4 signaling pathway inhibitor, were used as a positive control. C, specificity of cyclic peptides as demonstrated by TGF-β-induced α transcript levels. Active cyclic peptide inhibitors were tested in ST486 cell line. Data are shown for the four most active cyclic peptides. All peptides exhibiting down-regulation of germline ε transcription had no effect on TGF-β-induced α transcript levels.

**Functional Genetic Screen for Identification of Cyclic Peptide Inhibitors**—We then tested the utility of the retroviral S+4 library using a previously validated protocol to screen retroviral-based libraries for genetic effectors that inhibit IL-4 induction of sterile ε-germ line transcription in a B cell line. The screening cell line A5T4, which was derived from BJAB cells (Fig. 2A), was engineered to contain an IL-4-responsive 600-bp fragment of the ε promoter (Pe) driving the expression of the dual reporter HBEGF-2a-GFP. HB-EGF functions as the receptor for diphtheria toxin and confers high sensitivity to diphtheria toxin killing following IL-4 induction. Inhibitors that block IL-4 signaling thus block the expression of HB-EGF and survive diphtheria toxin killing selection. GFP is used to monitor IL-4 induction of the Pe by fluorescence. The cleaving site
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Cell lines were created by retrovirally infecting A5T4 cells with putative cyclic peptide inhibitors in the context of wild-type and mutant intein. Select peptides were purified by microbore C18 reversed phase chromatography from cell lysates and detected by MALDI-TOF mass spectrometry (13). In some cases 100 pmol of synthetic standards were spiked into uninfected cell lysates. The theoretical and observed MH+ masses are listed for each peptide. N/A, not available.

| Cyclic peptide | Uninfected A5T4 cells spiked in synthetic peptide (MH+) | A5T4 cells infected with wild-type intein (MH+ theor/obs) | A5T4 cells infected with mutant intein |
|----------------|--------------------------------------------------------|--------------------------------------------------------|----------------------------------|
| SARFV          | 561.36                                                 | 561.40/561.32                                          | None observed                    |
| SRVEI          | N/A                                                    | 585.34/585.34                                          | None observed                    |
| SFPWT          | 621.34                                                 | None observed                                          | None observed                    |
| SLNRI          | 584.41                                                 | 597.27/597.26                                          | None observed                    |
| SSLRW          | 630.43                                                 | 630.43/630.47                                          | None observed                    |
| SNPTF          | N/A                                                    | None observed                                          | None observed                    |
| SGADS          | N/A                                                    | None observed                                          | None observed                    |
| SVIEQ          | N/A                                                    | None observed                                          | None observed                    |
| SFGRS          | N/A                                                    | None observed                                          | None observed                    |
| SCCMR          | N/A                                                    | None observed                                          | None observed                    |

Intein splicing is dependent upon the presence of nucleophilic residues at the splice junctions (13). Therefore splicing-defective mutants were created by replacing three critical residues, underlined in Fig. 2A, with alanine residues. Western blot analysis of lysates from cells expressing the wild-type and mutant intein constructs demonstrated that the intein mutations dramatically reduced precursor protein processing in most cells (data not shown). As shown in Fig. 3B, the intein mutant version of SRVEI failed to inhibit e promoter activity, indicating that residues necessary for cyclization were correspondingly required for Pe inhibition.

The analysis of the remaining clones is shown in Fig. 3C. Of the 23 clones tested, eight clones produced no inhibitory phenotype (≥15% inhibition; representative negative peptide SS-CMR in Fig. 3C). Fifteen clones re-confirmed the inhibitory phenotype (≥20% inhibition), 13 of which represented unique sequences. Of these, eight library sequences (SRVEI, SWAQG, SGADS, SVIEQ, SDHSQ, SFGRS, SNIPQ, SNFFT) showed inhibition when presented by the active intein but not when presented by the mutant intein. Three peptides, SARFV, SFPTV, and SSLRW, when expressed in the mutant intein seemed to show a decrease in inhibitory activity, while the remaining two peptides, SLNRI and STGPR, were equally active when presented by both the wild-type and the mutant DnaB intein. Analogous to peptides presented by other protein scaffolds (9), these last five peptides may also be active when presented by the intein structure as a display scaffold. It is not known whether these peptides were active both as cyclic peptides and in the fused context with the intein protein.

To assess the sequence specificity of the peptide inhibitors, a subset of peptides were subjected to random alanine or tyrosine scanning mutagenesis. Of the three peptides tested, each displayed one or more positions that were sensitive to amino acid substitutions as indicated by a loss of IL-4/e promoter inhibitory activity (data not shown). A further indication of the sequence specificity of inhibitory peptide activity is provided by the following line of reasoning: (i) the overall complexity of the peptide library that was screened (2.7 × 10^5 primary transformants) represents ~27% of the theoretical complexity of the library (32 NNK codons for each of four positions) meaning that about 25% of all possible codon combinations have been sampled; (ii) the amino acids that comprise a given 4 amino acid peptide can be rearranged in twenty-four (four factorial) ways while still maintaining the amino acid composition of the peptide; (iii) therefore, for each active peptide sequence recovered, approximately five “scrambled” versions were present in the library yet failed to exhibit sufficient inhibitory activity to survive the selection process.

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TABLE I

Detection of cyclic peptides by mass spectrometry

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| SCCMR          | N/A                                                    | None observed                                          | None observed                    |
Cyclic Peptide Inhibition of Endogenous Germ Line \(\epsilon\) Transcription—Next, the effects of the putative cyclic peptides on endogenous germ line \(\epsilon\) transcription levels induced by IL-4 were determined using quantitative RT-PCR. To control for cell population variability, the \(\epsilon\) transcription levels were tested and compared for each population in the presence or absence of dox. Both the wild-type and mutant forms were examined and are presented in Fig. 4. Peptides that were positive for phenotype transfer, also showed varying degrees of inhibition of endogenous transcript. Generally, peptides identified as strong inhibitors by GFP reporter gene inhibition (Fig. 3B) also demonstrated the greatest decrease of endogenous transcript (Fig. 4A), although none were as potent as the known IL-4 signaling inhibitor SOCS1 (18). The effect of the mutant inteins (Fig. 4B) generally mirrored results from GFP reporter gene inhibition, with the exception of SNFTF, which in the mutant context generally mirrored results from GFP reporter gene inhibition, with the exception of SNFTF, which in the mutant context clearly blocked endogenous \(\epsilon\) transcription but did not block GFP reporter gene inhibition. None of the peptides isolated in the screen displayed overt toxicity (based on proliferation rates and FACS light scatter profiles), demonstrating that they are unlikely to be general transcription inhibitors. In addition, the germ line transcript specificity of the putative cyclic peptides was examined by using quantitative RT-PCR to measure TGF-\(\beta\)-induced germ line \(\alpha\) transcripts in ST486 cells. None of the peptide constructs blocked \(\alpha\) transcript production (Fig. 4C and data not shown).

Mass Spectrometry-based Confirmation of Cyclic Peptide Inhibitors—Finally, using the method to detect cyclic SRGDGWS (Fig. 1D), we examined cell lysates for the presence of selected cyclic peptides using MALDI-TOF mass spectrometry (Table I). For SRVEI, SARFV, SSLRW, and SNFTF, the results were consistent with production of the cyclic peptide from the active inteins. Cyclic SSLRW showed the highest intracellular concentrations and when its peak intensity was compared with that of its synthetic counterpart spiked into uninfected cell lysates, an estimated intracellular concentration of \(\sim 0.5 \mu M\) was calculated (data not shown). This is similar to levels of GFP-fused peptide library members in A549 and Jurkat cells (9). No cyclic peptide was observed for SFVTW, SLNRI, SGADS, SVIEQ, SFGRS, and SSCMR. This could be attributed to the fact that the intracellular concentrations may be below our MS detection capabilities. Alternatively, these peptides may be undergoing partial splicing and may be active through an intermediate lariat structure (13).

Conclusion—In the present work, we have demonstrated that: 1) bacterial inteins in a split configuration can function in the cytoplasm of mammalian cells to generate cyclic peptides, and 2) a functional screen can identify elements of a library capable of producing cyclic peptides that selectively inhibit endogenous \(\epsilon\) promoter activity. Of note, the frequency of occurrence of active cyclic peptides in this screen is over two orders of magnitude higher than for an analogous screen with linear 20-mer peptides fused to BFP (data not shown). Since cyclic peptide expression does not appear to be significantly higher than the BFP-fused linear peptides, it seems that a more rigid cyclic peptide structure allowing tighter binding may contribute to this effect. Alternatively, the small cyclic peptides may have better access to active sites or crevices in target protein surfaces or occluded areas in protein complexes. It will be interesting to explore our S+5 and S+6 libraries, which offer more diversity and structural complexity.

The identity of the binding targets for these active cyclic peptides is currently unknown. None of the peptide sequences are highly similar. Thus, individual peptides may act at different binding sites to alter the cellular phenotype. Elucidation of the mechanism by which these cyclic peptides inhibit IL-4 signaling should point us to critical, non-redundant sites for effective intervention of the IL-4 signaling pathway.

Our results demonstrate that functional screening for cyclic peptides in vivo in mammalian cells is feasible, scaleable, relatively efficient, and may be applicable to other functional screening strategies (19). Furthermore, the accessibility of NMR structure determination for the identified active cyclic peptides may provide starting points for drug development.

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