Chemistry & Biology, Vol. 10, 975–987, October, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2003.09.009

Cellular Localization and Antiproliferative Effect of Peptides Discovered from a Functional Screen of a Retrovirally Delivered Random Peptide Library

Yasumichi Hitoshi,[1,*] Tarikere Gururaja,[1] Denise M. Pearsall,[1] Wayne Lang,[1] Poonam Sharma,[1] Betty Huang,[1] Susan M. Catalano,[1] John McLaughlin,[1] Erlina Pali,[1] Beau Peelle,[1,3] Jorge Vialard,[2] Michel Janicot,[2] Walter Wouters,[2] Walter Luyten,[2,4] Mark K. Bennett,[1] Dave C. Anderson,[1,5] Donald G. Payan,[1] James B. Lorens,[1] Jacob Bogenberger,[1] and Susan Demo[1]

[1]Rigel Pharmaceuticals Inc.
South San Francisco, California 94080

[2]Johnson & Johnson Pharmaceutical Research and Development
B-2340 Beerse
Belgium

Summary

We have generated a random peptide library fused to GFP in a retroviral vector system and used this library to screen for peptides inhibiting tumor cell growth. Four unique peptide sequences were isolated that exhibited antiproliferative effects and that specifically localized to the plasma membrane and cytoplasmic granular compartments. Mutational analysis revealed critical residues in each peptide sequence and demonstrated a correlation between peptide subcellular localization and antiproliferative activity. Synthetic analogs of the peptides with poly-lysine internalization sequences, but not loss-of-function mutant peptides, competed for subcellular localization of the parent GFP-fused peptides. The synthetic peptides exhibited dose-dependent antiproliferative effects in tumor cells, while mutant peptides had no effect. Our screening approach using retrovirally expressed intracellular peptides enables identification of unique sequences with a specific biological function and with potential as therapeutics.

Introduction

The generation and screening of diverse sets of molecular structures is a central tenet of modern drug discovery. These molecules can bind specific sites on proteins, altering their normal function and causing a distinct cellular phenotype. An alternative approach that builds on similar principles is intracellular expression of dominant genetic effectors. These effectors, exemplified by peptides or mutant proteins, can also bind and inhibit the normal function of specific proteins. Libraries of randomly generated peptide sequences represent a vast source of biomolecular structures that can uniquely affect cell-signaling pathways. Large peptide libraries can be efficiently expressed in mammalian cells as free entities or covalently linked to reporter proteins that act as scaffolds. A small subset of peptide library members will have the ability to bind cellular macromolecules, thereby perturbing catalytic or binding functions and resulting in an altered cellular phenotype. Selection strategies capable of enriching for rare cells with a desired phenotypic change, in conjunction with efficient peptide library expression, allow for functional genetic screens that can uncover novel peptide sequences with potential as tools for understanding cellular signaling pathways or as direct starting points for development of therapeutics.

Retroviruses are ideal delivery vehicles for the stable expression of peptides in mammalian cells. Retroviruses can be produced at high titer, infect dividing cells with high efficiency [1, 2], and exhibit diverse tropism. Furthermore, because retroviral vectors are stably integrated into the host cell genome, the cognate peptide sequence can be readily retrieved and transferred to a naïve cell population once a specific phenotype is identified. Because of these versatile characteristics, retrovirally mediated functional screening approaches have been utilized for identification of physiologically relevant regulators of cell signaling in normal and diseased cells [3–8]. Inducible expression systems allow phenotypes in both the presence and absence of genetic effector expression to be directly compared within the same cells. Incorporation of inducible systems in functional screens can provide a strong advantage, especially in identification of cell cycle regulators. Among several inducible gene expression systems, the tetracycline (tet)-regulatable system has been successfully incorporated into several mammalian expression systems including those mediated by retroviruses.

Individual peptides or peptide libraries can be expressed as either free entities or as covalently linked fusions with scaffolding peptides or proteins. While free peptides are typically unstable in an intracellular environment, expression of peptide-scaffold protein fusions can increase stability and intracellular concentration of potentially phenotype-altering peptide sequences. Several protein scaffolds, including thioredoxin [9], green fluorescent protein (GFP) [8, 10], and Staphylococcal nuclease [11], have already been used for intracellular peptide display in yeast, bacteria, and mammalian cells. GFP in particular affords a significant advantage because cellular localization and expression levels can be monitored based on its intrinsic fluorescence while the scaffold itself has low toxicity, high stability (resulting in high peptide expression levels), and few specific known interactions [12, 13].

A particularly intriguing but challenging application of these functional genomic approaches is the identification of novel peptide inhibitors of cell proliferation. Alteration of the expression and activity of cell proliferation

*Correspondence: yhitoshi@rigel.com
[3] Present address: Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.
[4] Present address: PharmaDM, Ambachtenlaan 54D, B-3001 Heverlee, Belgium.
[5] Present address: Institute for Molecular Biology, University of Oregon, Eugene, Oregon 97403.

regulators is common in a variety of human diseases such as cancer, cardiovascular disease, and psoriasis where aberrant proliferation contributes to the pathology of the illness. Cell proliferation is tightly regulated by the activation and inactivation of an array of signaling proteins. The endogenous activities of these signaling pathway members can be blocked by disruption of their protein-protein interactions and/or catalytic activities. We have shown recently that intracellular expression of peptides derived from the cell cycle inhibitor p21 can mimic the proliferating cell nuclear antigen (PCNA) interaction and phenotypic effects of the native protein [12]. Thus, small peptides constitute powerful tools for inhibiting intracellular signaling cascades and offer an alternative to conventional small molecule-based therapeutics for the treatment of several diseases including cancer [14–16].

We have developed a screen for growth inhibition that utilizes tet-inducible expression (tet-off system) of a GFP-fused random peptide library. A strategy for selection of cells exhibiting an arrested or growth-deficient phenotype has been devised based on maintenance of staining intensity following vital dye treatment and resistance to retroviral delivery of a cytotoxic gene. Peptides identified by the screen exhibited unique cellular localization patterns and significant antiproliferative effects. Our screen for growth inhibitors using a GFP-fused peptide library is a novel approach to identify and validate several functional peptide inhibitors, which may provide useful tools for understanding signaling pathways the treatment of various diseases including cancer.

Results

Intracellular Screening for Antiproliferative Peptides

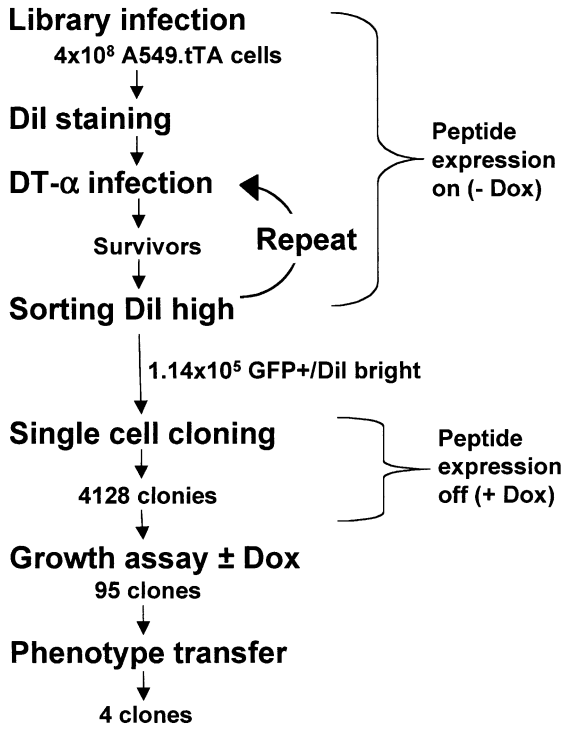
A dual selection scheme for the isolation of antiproliferative peptides was developed that entailed (i) positive selection for cells that fail to "dilute" the lipophilic cell membrane staining dye, DiI, through successive rounds of cell division and (ii) negative selection of cycling cells by infection with retrovirally encoded diphtheria toxin α chain (DT-α virus). Nondividing or slow growing cells, which remained DiI bright were easily distinguished and selected (without cell fixation) by fluorescence-activated cell sorting (FACS) while the preferential susceptibility of dividing cells to DT α virus infection (due to the requirement for nuclear envelope breakdown for efficient retroviral integration) efficiently eliminated cycling cells. A combination of these two selection methods was applied to a population of A549 cells expressing a GFP-fused peptide library to enrich for peptides that cause an antiproliferative phenotype. The screening strategy is depicted schematically in Figure 1A. Briefly, 4×10^8 A549.tTA cells were infected with the tet-inducible (tet-off) retroviral GFP-fused peptide library (TRA-GFP-C20 containing 1.1×10^9 independent inserts) and selected according to both the fluorescent intensity of DiI and resistance to infection with DT-α virus (see Experimental Procedures). The infection efficiency of the library was estimated at 10% based on GFP fluorescence. After two rounds of DT-α virus selection and DiI-based cell sorting, 1.1×10^5 cells were obtained and plated for single-cell cloning in the presence of doxycycline (Dox) to suppress GFP-peptide expression. More than 95% of tTA-mediated gene expression in A549.tTA cells was suppressed by 10 ng/ml Dox (data not shown). The cells that did not grow in Dox containing media (peptide off) probably due to spontaneous arrest or the effect of retrovirus insertion were eliminated during single-cell cloning. Following careful observation, 4128 colonies were picked and analyzed for their growth in both Dox-containing (peptide expression off) and Dox-free media (peptide expression on). Of these, 95 clones were identified that showed both reduction of cell growth in the absence of Dox and normal cell growth in the presence of Dox. The peptide-encoding retroviral inserts from these clones were amplified by PCR, recloned, and transduced into naïve A549.tTA cells to evaluate their effect on cell proliferation with the DiI staining intensity assay. Based on their ability to transfer phenotype, four peptides, 35, 38, 40, and 41, were identified as significant inhibitors of cell proliferation in A549.tTA cells.

As shown in Figure 1B, the inserts recovered from clones 35, 38, 40, and 41 encode 17-mer, 25-mer, 20-mer, and 25-mer peptides, respectively. Peptides 35 and 40 have an additional amino acid sequence (RPVRP) derived from the vector due to deletion of 1 nucleotide in the randomized region of the peptides. BLAST search analysis revealed that the four peptides do not have more than 50% total identity to any known sequences, indicating that the four peptides represent novel amino acid sequences.

Because the four peptides recovered in our functional genetic screen were selected, in part, based upon the fluorescence intensity of the cell tracker dye DiI, we utilized this assay to confirm the effect of each peptide individually on cell proliferation (Figure 2). Nondividing cells or slow growing cells remain DiI bright as a result of the decreased rate of cell division. Due to the efficiency of retroviral infection, transduction of A549.tTA cells with viruses encoding the GFP-peptide results in a mixed population of peptide-expressing (GFP positive) and uninfected cells (GFP negative). Thus, by FACS analysis, one can assess the effect of the peptide on proliferation by comparing the DiI intensity distribution of the GFP-positive population (gating on either the entire peak or a subpopulation consisting of the highest expressors) with that of the GFP-negative population in the same sample.

Negative controls, including destabilized GFP (dsGFP) and GFP-peptide 10 (an inactive member of the library), showed no significant difference in the DiI fluorescence intensity distribution between the GFP-positive and GFP-negative populations. (The very slight shift in DiI intensity for the highly GFP-positive subpopulation relative to the GFP-negative population is likely due to modest intrinsic GFP toxicity.) As a positive control, we utilized GFP fused to the PCNA binding C-terminal peptide of the tumor suppressor p21 (p21C; 24-mer, KRRQTSMT DFYHSKRRLIFSKRKP), a peptide previously shown to have a significant antiproliferative effect [12]. GFP-fused p21C-infected cells show higher DiI fluorescence in the GFP-positive versus -negative population. Similarly, cells infected with retroviruses encoding each of the four

A



B

#35: CGGCTCCGGAGAATATGTAGCGGCATTCTGCTCATCCGTAGGATATTGGGCATTTTCGTTAGGCCCGTGAGGCCCTAA
R L R R I C S G I L L I R R I L G I F V R P V R P

#38: ACTAGTGGGTTGCTGAAGCTGGTGCAGGCTAAGCGTAAGTGTTGTATTAGTTAG
T S G L L K L V Q A K R K C C I S

#40: CGTTGGGATCCGACGCGATTGCTGCGATTTCGGTTCCTCCGGATGCTAGTGAGGCGGAGTAGGCCCGTGAGGCCCTAA
R W D P T R L L R F R F L R M L V R R S R P V R P

#41: GGAAGGGGATGTATCTTTCGATGGAGGAGAGGCCTGCGGGGAATGATGAGACTATTTAAGTAG
G R G C I F R W R R G L R G M M R L F K

Figure 1. Isolation of the Peptides that Inhibit Tumor Cell Growth

(A) Flow chart for growth inhibitory peptide screening. 4×10^8 A549.tTA cells were infected with TRA-GFP-C20 and selected for resistance against infection of DT-α virus and fluorescent intensity of DiI (see Experimental Procedures). During two rounds of DT-α selection and cell sorting, peptide expression was on (−Dox). Following bulk selection, 1.1×10^5 cells were plated for single-cell cloning and peptide expression was suppressed (+Dox). 95 clones that showed both reduction of cell growth in the absence of Dox and normal cell growth in presence of Dox were identified from 4128 clones initially selected. The retroviral DNA inserts encoding peptides were recloned and transduced into naive A549.tTA cells to confirm their antiproliferative effect based on the fluorescent intensity of DiI.

(B) Nucleotide and amino acid sequences of 35, 38, 40, and 41. The amino acid sequences and in-frame stop codons in each peptide are indicated as bold letters. The vector sequence is underlined.

peptides recovered from the screen (35, 38, 40, and 41) exhibited higher DiI fluorescence in the GFP-positive population, indicative of their inhibition of cell proliferation. Furthermore, the antiproliferative effect (maintenance of DiI staining intensity) of each of the four peptides was more pronounced in the highly GFP-positive subpopulation, indicating that their activity is dose dependent. Based on the intensity of GFP fluorescence, two of the peptides (35 and 40), appeared to be expressed at 10-fold lower levels than the other two peptides, suggesting that they may represent more potent inhibitors of proliferation. All four peptides had a similar antiproliferative effect in HeLa cells (cervical carcinoma cells, p53 deficient), Colo205 cells (colon cancer cells, p53 mutant), H1299 (lung cancer cells, p53 null), MCF7 (breast cancer cells, p53 wild-type), and normal human mammary gland epithelial cells (p53 wild-type) (data not shown).

The effects of the four peptides on the cell cycle of A549.tTA cells were also analyzed (data not shown). Cells expressing each peptide or dsGFP were stained with Hoechst 33258 to allow estimation of DNA content. Cells infected with either 38 or 41 appeared to be arrested in G0/G1 because cells in the S and G2/M phases were significantly reduced. Cells infected with either 35 or 40 showed no significant change in the DNA content profile. None of the GFP-fused peptides induced massive cell death as assessed by either direct observation or appearance of a sub-G1 population (data not shown).

Because the peptides active in the functional screen were fused to GFP, we sought to study their localization in A549.tTA cells using confocal microscopy (Figure 3A). The four antiproliferative peptides were predominantly localized to the cytoplasm, while dsGFP alone or an inactive peptide (10, data not shown) was detected in both the cytoplasm and the nucleus. Of the four antiproliferative peptides, three (38, 40, and 41) were concentrated both at the plasma membrane and in association with cytoplasmic granular structures, while the majority of the fourth peptide (35) was localized to cytoplasmic granules but not to the plasma membrane. Peptides 35 and 38, and to a lesser extent peptide 41, exhibited nuclear exclusion.

A colocalization study was performed with GFP-fused

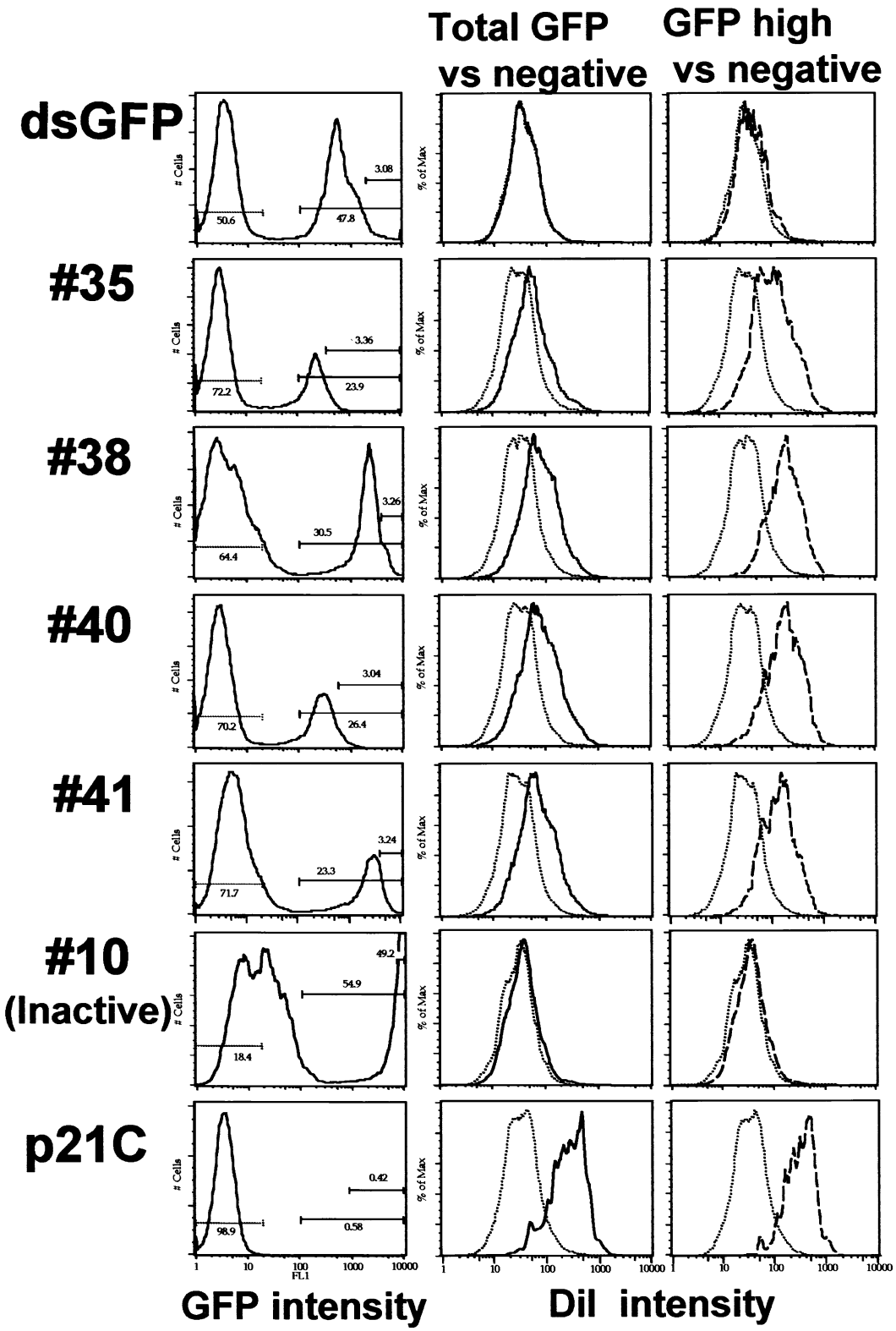
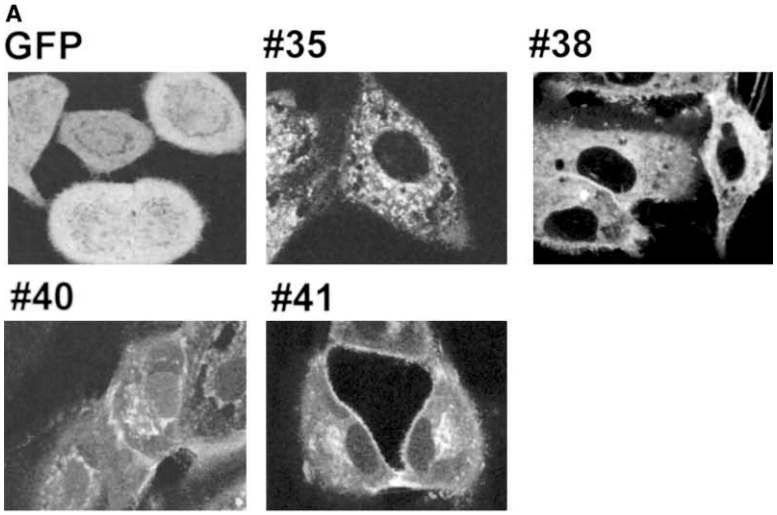


Figure 2. Cell Tracker Assay for Four Antiproliferative Peptides, 35, 38, 40, and 41

Fluorescent intensity of GFP and DiI in A549.tTA cells expressing peptides 35, 38, 40, 41, 10 (inactive peptide), dsGFP, or C-terminal peptide of the CDK inhibitor p21 was analyzed using flow cytometry. DiI intensity of GFP-positive population (solid line), GFP high population (dashed line), and GFP-negative population (dotted line) is shown as histograms (middle, right). Gates for the three populations are indicated in the GFP histograms (left).



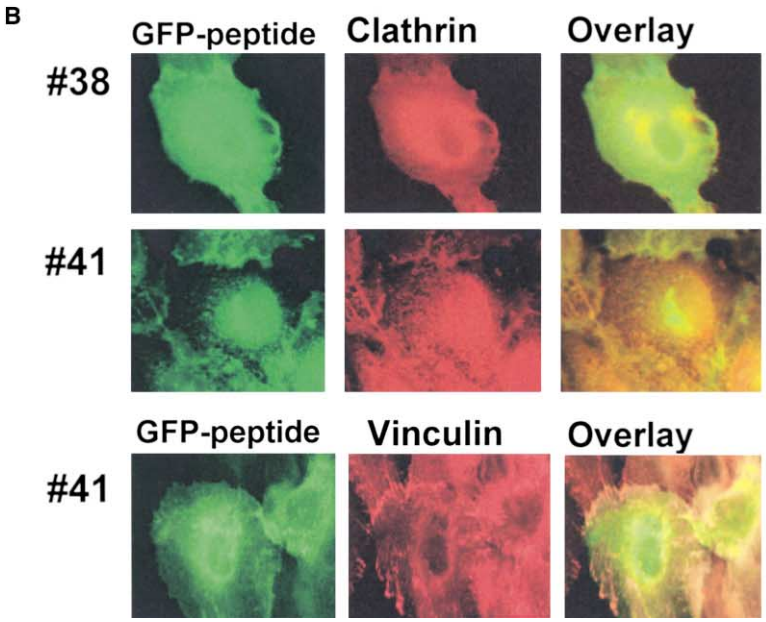


Figure 3. Cytoplasmic Localization of Four Antiproliferative Peptides

(A) Cellular localization of GFP-fused peptides 35, 38, 40, and 41. A549.tTA cells expressing GFP-fused peptides 35, 38, 40, and 41 were plated on coverslips and analyzed with a confocal scanning microscope (Leica Mycrosystems, Inc., Bannockburn, IL).

(B) Localization of clathrin and GFP-fused peptides. A549.tTA cells expressing peptides 38 or 41 were costained with anti-GFP antibody and anti-clathrin antibody or anti-vinculin antibody. Images were observed by a fluorescent microscopy. The optical sections were acquired through the green and red channels for GFP and clathrin, respectively.

peptides 38 and 41. Both peptides had a similar localization to the plasma membrane (most pronounced at cell-cell contact areas) and to cytoplasmic granular structures. We examined costaining with several membrane- and cytoskeleton-localized proteins. Our analysis revealed that clathrin, which also localizes to the plasma membrane and cytoplasmic granular structures, overlaps with the localization of peptides 38 and 41 (Figure 3B). We did not see colocalization of peptides 38 and 41 with mitochondrial markers, Golgi markers, actin, tublin, annexin II, vinculin, plakoglobin, desmoplakin, Ras, or transferrin-labeled granules (data not shown except for vinculin). Although peptide 41 contained a leucine-rich motif associated with nuclear exclusion mediated by exportin 1 (see below), we did not observe colocalization with exportin-1 and localization of the peptide was resistant to the exportin 1 inhibitor Leptomycin B (data not shown).

Mutational Analysis of Peptide 38

Peptide 38 contains a carboxy-terminal CAAX box motif that typically serves as a signal for posttranslational prenylation and results in membrane attachment of the prenylated protein product [17–19]. In the prenylation reaction, a 15 carbon farnesyl or 20 carbon geranylgeranyl isoprenoid is attached to the CAAX motif Cys residue by a farnesyl transferase or geranylgeranyl transferase, respectively. Interestingly, the carboxy-terminal half of peptide 38 is nearly identical to the 17 carboxy-terminal residues of cell division cycle 42 isoform 2, (CDC42C;

Peptide	Sequence
#38	TSGLLKLVQAKRKCCIS
CDC42C	AALEPPETQPKRKCCIF
#38ΔN(1-8)	QAKRKCCIS
#38ΔN(1-13)	CCIS
#38(T1A)	ASGLLKLVQAKRKCCIS
#38(S2A)	TAGLLKLVQAKRKCCIS
#38(G3A)	TSALLKLVQAKRKCCIS
#38(L4A)	TSGALKLVQAKRKCCIS
#38(L5A)	TSGLAKLVQAKRKCCIS
#38(K6A)	TSGLLALVQAKRKCCIS
#38(L7A)	TSGLLKAVQAKRKCCIS
#38(V8A)	TSGLLKLAQAKRKCCIS
#38(C14A)	TSGLLKLVQAKRKACIS
#35	RLRRICSGILLIRRILGIFVRPVRP
#35(I18A)	RLRRICSGILLIRRILGAFVRPVRP
#35(L16A/I18A)	RLRRICSGILLIRRIAGAFVRPVRP
#35(I12A/L16A/I18A)	RLRRICSGILLARRIAGAFVRPVRP
#40	RWDPTRLLRFRFLRMLVRRSRPVRP
#40(M15A)	RWDPTRLLRFRFLRALVRRSRPVRP
#40(L13A/M15A)	RWDPTRLLRFRFARALVRRSRPVRP
#40(F10A/L13A/M15A)	RWDPTRLLRARFARALVRRSRPVRP
#41	GRGCIFRWRRGLRGMMRLFK
#41(L18A)	GRGCIFRWRRGLRGMMRAFK
#41(M16A/L18A)	GRGCIFRWRRGLRGMARAFK
#41(L12A/M16A/L18A)	GRGCIFRWRRGARGMARAFK

```
                        1   2   3 4
HIV1 REV                LPP-L-ERLTLD
MAPKK                   LQKKL-EELELD
HTLV1 Rex               LSAQLYSSLSLD
Hdm-2                   ISLSFDESLALC
PKI                     LALKL-AGLDIN
#35              RLRRICSGILL-IRRILGIFVRPVRP
#40                RWDPTRLLR-F-RFLRMLVRRSRPVRP
#41               GRGCIFRWRRGLRGMMRLFK
```

Figure 4. Amino Acid Sequences of GFP-Fused Peptides 35, 38, 40, 41 and Their Mutants

Mutated residues or nonidentical residues of the C terminus of cell division cycle 42 isoform 2, CDC42C, (amino acids 175–191, gi|16357472|ref|NP_426359.1|) to 38 are underlined. Amino acid sequences of 35, 40, and 41 are aligned with leucine-rich motifs of HIV-1 Rev (amino acids 75–84, gi|16118296|gb|AAL12672.1|), HTLV-1 Rex (amino acids 82–93, gi|7963889|gb|AAF71372.1|), mitogen-activated protein kinase kinase 1, MAPKK, (amino acids 33–43, gi|5579478|ref|NP_002746.1|), human homolog of mouse double minute 2, Hdm-2, (amino acids 189–200, gi|4505137|ref|NP_002383.1|), and protein kinase inhibitor, PKI (amino acids 38–48, gi|203271|gb|AAA40867.1|).

Figure 4) (gi|16357472|ref|NP_426359.1|), a Rho-family GTPase that has been shown to be geranylgeranylated [20].

To investigate the relative importance of the CAAX motif and other sequence elements within peptide 38, we evaluated the localization and antiproliferative activity of a variety of peptide 38-derived deletion and alanine point mutants (Figure 4). As shown in Figures 5A and 5B, mutation of the CAAX motif Cys residue to Ala (C14A) resulted in loss of both antiproliferative activity and plasma membrane/granular localization. Furthermore, although all peptides retaining an intact CAAX motif (including GFP-CDC42 peptide) exhibited at least some plasma membrane/granular localization similar to wild-type peptide 38, only a subset of peptide 38 mutants demonstrated an antiproliferative phenotype. These observations indicate that the CAAX motif is (i) necessary and sufficient for membrane localization and (ii) necessary but not sufficient for antiproliferative activity. An additional important contribution to the antiproliferative activity of peptide 38 is made by amino-terminal sequence components. Deletion of the amino-terminal half of peptide 38 (ΔN1-8), leaving the carboxy-terminal CCIS and localization intact, results in a strong reduction of antiproliferative activity while alanine point mutants within the first 8 residues of peptide 38 exhibit either a reduced (L4A, L5A, K6A, L7A) or enhanced (T1A, S2A) cell proliferation phenotype. A portion of the membrane localization of peptide 38 may also be provided by non-CAAX elements since deletion of all but the CCIS sequence (ΔN1-13) reduced membrane association while the K6A point mutant displayed enhanced granular and reduced plasma membrane localization. The GFP fluorescence intensity of all GFP-fused peptide 38 mutants was similar to that of the wild-type peptide (data not shown). Together, these observations indicate that the antiproliferative activity of peptide 38 requires both an intact carboxy-terminal CAAX motif for membrane localization as well as critical sequence elements within the first 8 amino acids at the amino terminus.

To further evaluate the proposed importance of peptide 38 prenylation for membrane localization, A549.tTA cells expressing peptides 38 and 41 (a peptide lacking the CAAX motif) were treated with a peptidomimetic farnesyltransferase specific inhibitor, L-744,832, for 48 hr. As shown in Figure 5C, membrane localization of peptide 38, but not 41, was completely abolished in the presence of 2 μM L-744,832, indicating that localization

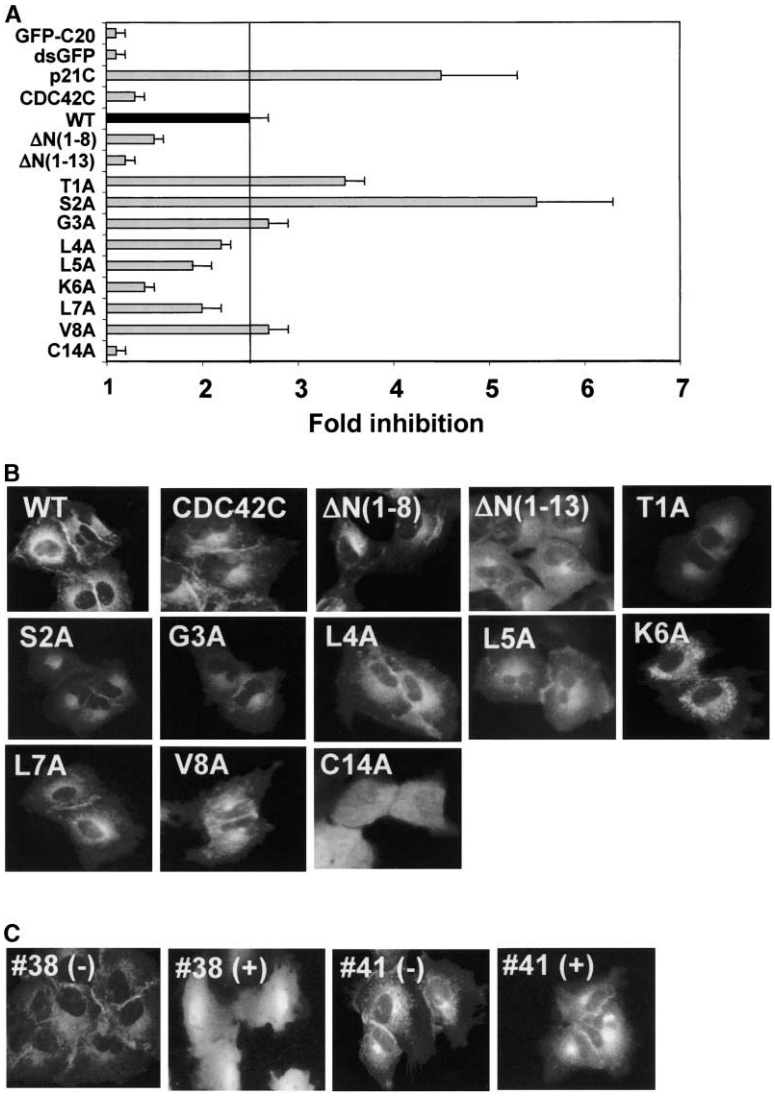


Figure 5. Mutational Analysis of Peptide 38

(A) A549.tTA cells were infected with retroviruses encoding GFP-fused peptides (38 and its mutants) and stained with the cell tracker dye, DiI. Fluorescent intensity of GFP and DiI was examined by flow cytometry. A "fold inhibition" of proliferation was determined by the ratio of mean DiI fluorescent intensity for the GFP-positive population relative to that of the GFP-negative population. Fold inhibition is shown as mean ± SE in duplicate. As controls, the entire GFP-fused peptide library, dsGFP- and GFP-fused p21C-expressing A549.tTA cells were used.

(B) Localization of GFP-fused peptides was examined by fluorescence microscopy. The images were captured with a CCD camera.

(C) Farnesyltransferase-dependent localization of 38. A549.tTA cells expressing 38 and 41 were plated on coverslips and incubated with 2 μM L-744,832 for 48 hr (+) or without L-744,832 (−). Localization of GFP-fused peptides was examined by flourescence microscopy and images were captured with a CCD camera.

of peptide 38 is farnesyltransferase dependent. Consistent with the reported geranylgeranyl modification of CDC42 [20], localization of the 17 carboxy-terminal residues of the CAAX box peptide from CDC42 (CDC42C) was unaffected by L-744,832 (data not shown).

Mutational Analysis of Peptides 35, 40, and 41

In contrast to peptide 38, peptides 35, 40, and 41 are significantly enriched for arginine and hydrophobic amino acids such as leucine, isoleucine, methionine, and phenylalanine relative to 41 peptides randomly picked from the library or the theoretical occurrence of these amino acids based on codon frequency (data not shown). Interestingly, the sequence of peptides 35, 40, and 41 could be aligned with a leucine-rich motif found in HIV-1 Rev, HTLV-1 Rex, mitogen-activated protein kinase kinase 1 (MAPKK), human homolog of mouse double minute 2 (Hdm-2), and protein kinase A inhibitor (PKI), when other hydrophobic residues such as isoleucine, methionine, phenylalanine, and tryptophan are allowed to replace some leucines (Figure 4). This leucine-rich motif is characterized by three leucines separated by 2–3 other residues, and a fourth leucine separated by a single residue. These motifs are reported to mediate nuclear exclusion through recognition and transport out of the nucleus by a complex including exportin-1 [21–23].

To evaluate the importance of the leucine-rich sequence motifs for the localization and the antiproliferative effects of peptides 35, 40, and 41, alanine mutants were constructed at one or more of the positions occupied by hydrophobic residues within the motif. Single, double, and triple mutants were constructed for each peptide (corresponding to positions 4, 3+4, and 2+3+4 in the motif; see Figure 4). Each mutant was evaluated for antiproliferative activity in the DiI staining intensity assay and for GFP localization by fluorescence microscopy (Figure 6). For peptides 40 and 41, single, double, and triple mutants exhibited progressively reduced localization at the plasma membrane and granular cytoplasmic structures, and antiproliferative activity. The effects on proliferation appeared to be more pronounced with peptide 41 mutants. However, it is difficult to deter-

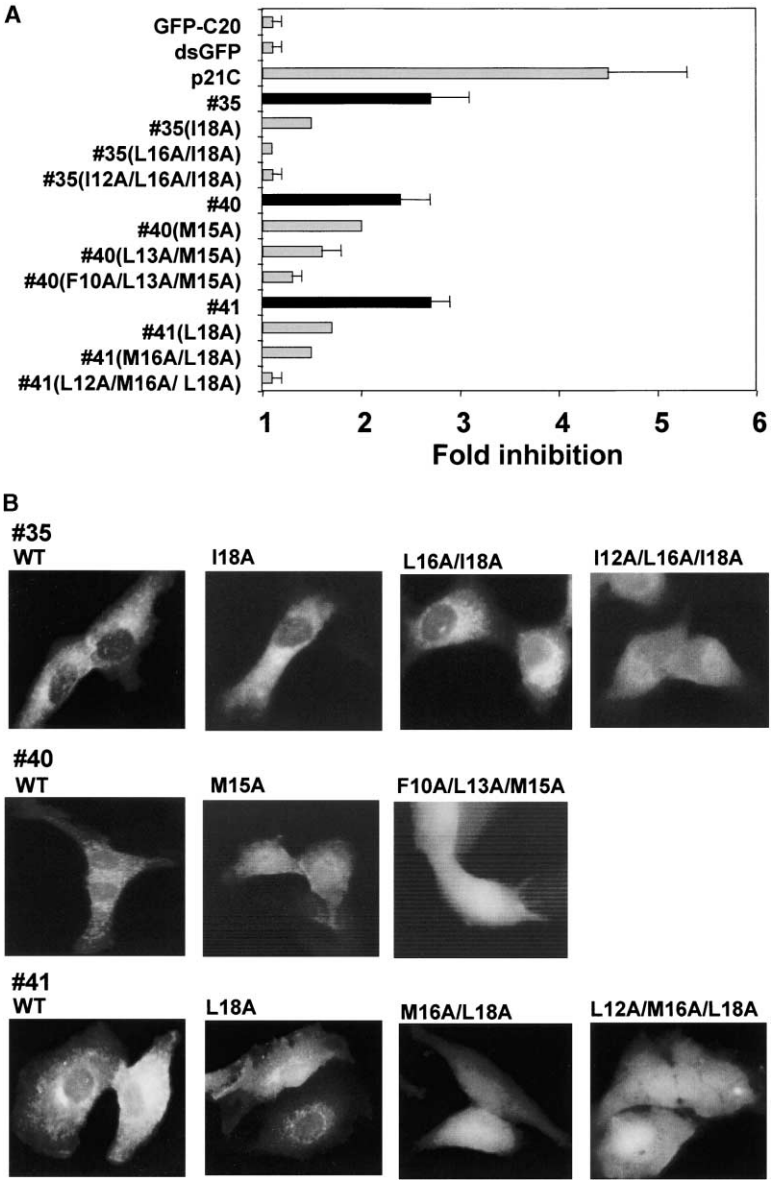


Figure 6. Mutational Analysis of Peptides 35, 40, and 41

(A) A549.tTA cells expressing GFP-fused peptides (35, 40, 41, and their mutants) were stained with DiI. Fluorescent intensity of GFP and DiI was examined by flow cytometry. A fold inhibition of proliferation was obtained as described above (Figure 5, legend). Fold inhibition is shown as mean ± SE in duplicate. As controls, the entire GFP-fused peptide library, dsGFP- and GFP-fused p21C-expressing A549.tTA cells were used.

(B) Localization of GFP-fused peptides was examined by fluorescence microscopy. The images were captured with a CCD camera.

mine the precise correlation between sequence, localization, and antiproliferative activity due to the nonquantitative nature of fluorescence microscopy and the observation that the mutant peptides were expressed at higher levels (based on GFP intensity) relative to the wild-type peptides (2-fold for peptide 40 and 2- to 10-fold for peptide 41) (data not shown). For peptide 35, the single (I18A) and double (L16A/I18A) mutants retained localization to cytoplasmic membranes and nuclear exclusion similar to the wild-type peptide, while the triple mutant (I12A/L16A/I18A) exhibited reduced membrane association and nuclear exclusion. In addition, cells expressing L16A/I18A or I12A/L16A/I18A lost their antiproliferative effect. The fluorescence intensity of all GFP-fused peptide 35 mutants was similar to that of the wild-type peptide (data not shown). These results with peptide 35, most notably the double mutant L16A/I18A, indicate that subcellular localization and antiproliferative activity are poorly correlated. Furthermore, the role of the leucine-rich motif in maintaining nuclear exclusion appears to be less critical for peptide 35 relative to peptides 40 and 41.

In order to examine whether the GFP protein scaffold is required for the antiproliferative effect of the peptides identified in our genetic screen, we synthesized peptides 40 and 41 (and corresponding mutants in which the leucine-rich motif was replaced with four alanines; shown in Figure 7A). Because polybasic peptides can be efficiently internalized by cells [24–26], the peptides were fused with 7 Lys residues (K7) via a linker (GGEEAAKA) at their amino terminus (K7_40, K7_40M, K7_41, and K7_41M). FITC-labeled versions of the synthetic peptides were also used for localization studies. The fluorescent peptides localized not only to the plasma membrane and cytoplasm but also to the nucleus, indicating efficient uptake of these peptides by the cells (data not

A

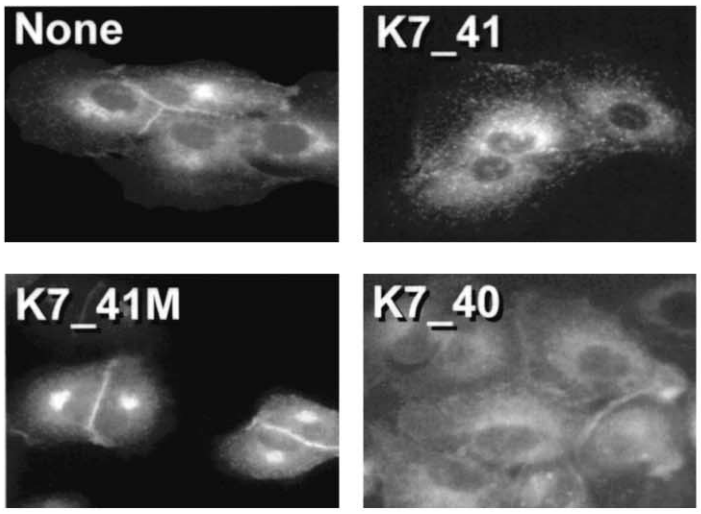
K7_40 : KKKKKKKGGEEAAKARWDPTRLLRFRFLRMLVRRSRPVRP

K7_40M: KKKKKKKGGEEAAKARWDPTRALRARFARALVRRSRPVRP

K7_41 : KKKKKKKGGEEAAKAGRGCIFRWRRGLRGMMRLFK

K7_41M: KKKKKKKGGEEAAKAGRGCIFRARRGARGMARAFK

B



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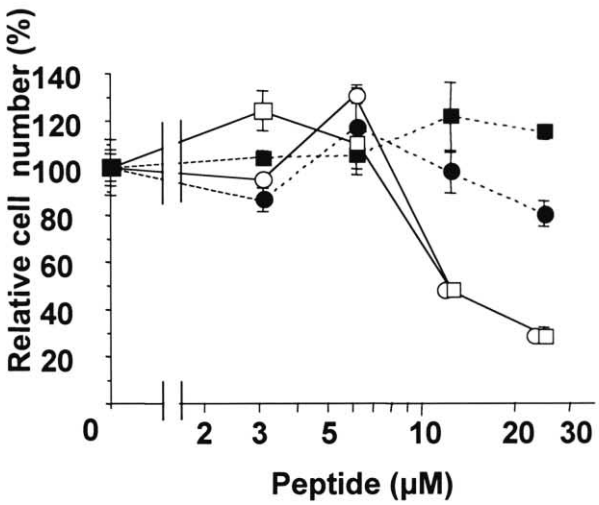

Figure 7. Evaluation of Synthetic Peptides

(A) Synthetic peptides 40 and 41 and their mutant sequences. The peptides were fused with seven Lys (K7) via a linker (GGEEAAKA) at the amino terminus (K7_40, K7_40M, K7_41, and K7_41M).

(B) Displacement of GFP localization with synthetic peptides. A549.tTA cells expressing GFP-fused peptide 41 were plated on coverslips and incubated with 100 μM of K7_41, K7_41M, K7_40, or no peptide for 5 hr, and GFP localization was analyzed by fluorescence microscopy. The images were captured with a CCD camera.

(C) Antiproliferative effect of synthetic peptides. A549 cells were incubated with peptides K7_40, K7_40M, K7_41, and K7_41M at a final concentration ranging from 0.8 to 25 μM in triplicate for 24 hr. The number of nuclei in each well was counted using a Zeiss Axiovert microscope, UV filter set, and Photometrics camera. The number of nuclei was normalized to that of untreated samples and is shown as % mean (symbol) ± % SD (bar). The mean percentage of nuclei is indicated as relative cell number in the graph. Open square, K7_40; closed square, K7_40M; open circle, K7_41; closed circle, K7_41M.

shown). Because of the nature of polybasic peptides [24, 25], they were predominantly transported to the nucleus and nucleoli. We did not see a significant difference in the localization of the K7_40 and K7_41 peptides or between the wild-type and mutant peptides.

If these synthetic peptides interact with the same protein target as the GFP-fused peptides, they should compete for cellular localization with the GFP-fused peptides. Because GFP-fused peptides 38 and 41 were similarly concentrated at the plasma membrane and granular cytoplasmic structures, especially at the cell-cell contact area, we examined whether K7_41 could displace membrane localization of the GFP-fused peptides. A549.tTA cells expressing GFP-fused peptide 41 were incubated with 100 μM of K7_41, K7_41M, and K7_40 for 5 hr and GFP localization was analyzed using fluorescence microscopy (Figure 7B). A549 cells expressing GFP-fused peptide 41 treated with K7_41 peptide showed more pronounced cytoplasmic granular localization of GFP and loss of GFP localization in the cell-cell contact area. Importantly, these changes in GFP peptide 41 localization were not observed in cells

treated with K7_41M or K7_40. Surprisingly, similar changes were observed when A549.tTA cells expressing GFP-fused peptide 38 were cultured with K7_41, but not K7_41M or K7_40 (data not shown). These results indicate that K7_41 specifically perturbs the plasma membrane localization of GFP-fused peptides 41 and 38.

In order to determine whether the synthetic peptides directly affected cell proliferation, A549 cells were treated with K7_40, K7_40M, K7_41, and K7_41M for 24 hr. The cells were fixed and stained with the DNA dye DAPI and the number of nuclei present in each well was quantified with an automated imaging method (as described in Experimental Procedures). Both K7_40 and K7_41 elicited a dose-dependent decrease in the number of cells with a midpoint concentration of 10–15 μM, while K7_40M and K7_41M showed no significant reduction in cell number (shown in Figure 7C). Together, these results suggest that synthetic versions of the peptides isolated in our functional genetic screen (appended with an internalization sequence) can both displace the GFP-fused functional peptides from their intracellular target(s) and mimic the effect of the GFP-fused peptides on cell proliferation.

Discussion

In this paper, we have demonstrated the ability to identify unique antiproliferative peptides from a random library expressed in a human lung carcinoma cell line. A FACS-based genetic selection scheme was devised based on cell tracker dye (DiI) accumulation and cell survival after infection of the toxic retrovirus. Incorporation of the tet-inducible system in our screen was also a key element for identification of the peptides. Hence we established a new approach to identify inhibitors of cell proliferation, which was quite challenging. According to the nature of our screening, we expected to identify peptides that confer an antiproliferative phenotype but do not exhibit cytotoxic effects. Indeed, all four peptides recovered from the screen and characterized in the present study were antiproliferative in the DiI accumulation assay and did not induce cell death. Each of the GFP-fused peptides identified in this study showed an antiproliferative effect in p53 wild-type cells (MCF7, A549, and normal human mammary epithelial cells), as well as p53-deficient cell lines (HeLa, Colo205, and H1299). Thus, this inhibition appears to be independent of p53 status, and these peptides do not show an absolute distinction between tumor and normal cells. Interestingly, the four antiproliferative peptides showed specific localization to the plasma membrane or granular cytoplasmic structures. One peptide (38) contains a CAAX box motif while the other three (35, 40, and 41) have related leucine-rich motifs. Our deletion and alanine-scanning mutational analysis indicated that localization of the peptides is crucial for optimal antiproliferative activity and that the CAAX box and leucine-rich motifs play a key role in effective localization of the peptides. Importantly, synthetic analogs of two peptides (40 and 41) exhibited dose-dependent antiproliferative effects that were not observed with their corresponding mutant peptides.

Peptide 38 has a functional CAAX box motif at its carboxyl terminus; membrane localization is disrupted by mutation of the critical Cys residue or upon addition of a farnesyl transferase inhibitor. Notably, deletion of the amino-terminal 13 residues of peptide 38 (leaving only the CAAX motif) also alters its localization. Thus, peptide 38 is most likely anchored to cellular membranes by prenylation via a mechanism that is, in part, dependent on the presence of specific amino-terminal residues in an adjacent basic region. The requirement of a similar basic region has been documented for plasma membrane targeting of p21K-ras(B) [18]. Deletion of the amino-terminal 8 residues of peptide 38 retains its membrane localization, but not its DiI accumulation-based antiproliferative activity, indicating that localization by farnesylation is necessary but not sufficient for the observed inhibition of cell growth. The K6A mutant also showed changes in localization associated with a loss of antiproliferative activity, indicating this residue also plays an important role in the localization and function of peptide 38. Taken together, GFP-fused peptide 38 likely exerts an antiproliferative effect through interaction with a target molecule(s) in the cell membrane rather than by inhibiting a farnesyl transferase.

Interestingly, the antiproliferative effect of the GFP-fused peptide 38S2A mutant was enhanced and exhibited potency similar to that of the PCNA binding GFP-p21C. This may be due to the increased affinity or accessibility of the peptide to the target protein. It is important to note that through systematic mutagenesis, one could optimize the peptide's antiproliferative potency. Such an optimized peptide could be useful tool for validating candidate target proteins.

GFP-fused peptide 35-, 40-, and 41-derived alanine mutants were constructed and tested for both localization and function. Multiple mutations in the leucine-rich motif resulted in loss of the antiproliferative effect and alteration of cellular localization. However, the effects on antiproliferative activity did not always coincide with effects on cellular localization (particularly for peptide 35). These results suggest that the leucine-rich motif may be involved in cellular localization to membrane structures or the plasma membrane and enhance the antiproliferative effect by increasing the local concentration of GFP-fused peptides. Peptide 40 and 41 exhibited apparent nuclear exclusion by standard fluorescence microscopy (Figure 6B). However, confocal microscopy indicated that peptides 40 and 41 were not completely nuclear excluded (Figure 3A) but are highly concentrated at the plasma membrane and cytoplasmic granular structures.

Identification of the protein targets of the antiproliferative peptides is a very important next step, because the protein would be already validated as a target for antiproliferative drugs, whether or not the drugs were based on the peptides isolated from this screen. There are several advantages of this peptide-based approach. (1) Since peptides can be expressed as GFP-fused proteins, colocalization study with antibodies against known molecules could be easily performed [8]. (2) Simple immunoprecipitation experiments using anti-GFP antibody could isolate candidate target proteins [12]. (3) Ease of procuring synthetic peptides and their immobilization

on beads could allow us to perform affinity extraction of target proteins from cell extracts and their identification via recent state-of-the-art proteomics strategies [12, 27]. (4) Gain-of-function mutants and loss-of-function mutants are easily identified. These mutants could functionally verify target proteins. We also developed a novel method to identify the functional protein targets of active peptides using a yeast two-hybrid (YTH) approach recently (T. Kinsella, E. Masuda, M.K.B., B.H., J. Warner, T. Kinoshita, A. Martinez, and P. Achacoso, unpublished data). Currently three approaches, immunoprecipitation, affinity extraction, and YTH, are underway in order to find the target proteins

Because GFP-fused peptides 38 and 41 were localized to the plasma membrane and cytoplasmic granule structures, we tested several membrane-associated proteins for colocalization. We found that GFP-fused 38 and 41 colocalized with clathrin, but not other membrane associated markers. Clathrin is a major protein component of coated vesicles and coated pits located on the cytoplasmic face of intracellular membrane structures [28–31]. These specialized structures are involved in the intracellular trafficking of receptors and endocytosis of a variety of macromolecules. The antiproliferative effect of GFP-fused peptides 38 and 41 may be a result of their role in altering the constitutive trafficking process of a variety of transmembrane proteins.

The synthetic peptides K7_40 and K7_41, but not their corresponding mutants, K7_40M and K7_41M, clearly induced a dose-dependent inhibition of cell proliferation. This indicated that the GFP protein scaffold is not required for the antiproliferative effect of the peptides, suggesting that a GFP-peptide library can be effectively screened to isolate such peptides. FITC-labeled version of both the wild-type and mutant peptides displayed plasma membrane, and perinuclear and nuclear localization. These results suggest that the synthetic peptides are effectively internalized through the cell membrane and do not require the GFP scaffold for their antiproliferative effect. However, we did not observe a significant difference in the localization of the wild-type and the mutant, suggesting that these peptides bind several targets not related to their antiproliferative effect. This nonfunctional target binding may be attributed to the hepta-lysine sequence. Hence, localization of fluorescently labeled K7_40 and K7_41 is distinct but overlapping with that of the corresponding GFP-fused peptides. Importantly, the K7_41 synthetic peptide displaced the plasma membrane localization of GFP-fused peptides 41 and 38, while the displacement was not observed in cells treated with K7_41M or K7_40. These results indicate that (i) K7_41 interacts with the same target(s) as GFP-fused peptide 41 and likely exerts its antiproliferative effect by virtue of this interaction, and (ii) that GFP-fused peptides 38 and 41 may function through common target molecules or may be closely related in a common pathway suppressing cell growth. The prospect that peptides 38 and 41 act by similar mechanisms is further supported by the fact that GFP-fused 38 and 41 were concentrated similarly at the plasma membrane (especially at areas of cell-cell contact) and colocalized with clathrin, and that overexpression of both GFP-fused peptides showed accumulation of cells at G0/G1.

Functional genetic screening approaches represent an efficient means for identifying and validating novel small peptide inhibitors. Several methods for "presenting" peptide libraries have been described, including the production of linear scaffold-peptide fusions as in the present study and methods that constrain the peptide structure. Constraining peptides by tethering both ends within the structure of a larger protein reduces the entropic cost of binding, thus improving the chance of isolating a high-affinity interactor. In this context, we have recently exemplified this concept of constrained random peptide libraries delivered by retroviral vectors [4, 5, 8]. Furthermore, as a proof-of-concept we have successfully delivered random cyclic peptide libraries utilizing retroviral technology and recently demonstrated that functional cyclic peptides can be identified as inhibitors of IL-4 receptor-mediated signaling cascade [7]. However, the use of constrained peptide libraries may prevent binding to potentially important targets owing to the decreased internal flexibility of the peptides. It is noteworthy that increased flexibility affords more structural variability, thus increasing the probability of identifying a conformational state capable of interacting with a target structure. As an example, a 20 amino acid peptide derived from the C terminus of the cell cycle inhibitor p21 (p21C) inhibits in vitro replication of simian virus 40 DNA and binds to PCNA in whole-cell extracts [14]. Intracellular expression of p21C fused to the C terminus of GFP also binds PCNA and induces a potent cell cycle arrest [12, 13]. Thus, as exemplified by the present study, a GFP-fused peptide library is a viable method to identify functional linear peptide inhibitors. An important advantage of the random peptide screening strategy is the novelty of the sequences that are isolated. Such novel sequences represent unique tools to probe important disease-relevant signaling pathways and have the potential, particularly in the case of cyclic peptides, to serve as starting points for the development of peptide-derived therapeutics. Since peptides could serve as a starting point of small molecule drugs, further studies including identifying the minimal antiproliferative peptides via truncation analysis and obtaining more potent peptides by introducing nonnatural amino acids would be very important.

Significance

The creation of diverse biomolecule libraries and analysis of their effects on cell physiology is a powerful approach to elucidating important signaling pathways in normal and diseased cells. Libraries of randomly generated peptide sequences represent a vast source of novel structural entities. Large peptide libraries can be efficiently delivered and expressed in mammalian cells by retroviral vectors. Cells expressing random, free, or constrained peptide structures can be screened for specific peptide-induced phenotypes. We generated a random peptide library fused to the C terminus of GFP and sought to identify peptides that can inhibit the growth of lung carcinoma cells. A novel FACS-based genetic screen was devised to effectively enrich for nonproliferating cells, through detection of nondi-

viding cells with the cell tracker dye, DiI, and reduction of cycling cells by infection with retrovirally encoded diphtheria toxin α chain. Four unique peptide sequences were isolated that exhibited antiproliferative effects in several cell types. These peptides demonstrated specific localization to the plasma membrane and cytoplasmic granular compartments. One peptide contained a CAAX box motif, which is important for its membrane localization and growth inhibitory activity. Three other peptides contained a leucine-rich motif, similar to a known nuclear exclusion motif. Mutational analysis revealed critical residues in each of the peptide sequences and demonstrated a correlation between peptide subcellular localization and antiproliferative activity. Synthetic analogs of the peptides with poly-lysine internalization sequences, but not loss-of-function mutants, competed for subcellular localization of the GFP-scaffolded peptides. The synthetic peptides exhibited dose-dependent antiproliferative effects on tumor cell lines, while mutant peptides had no effect. Our results demonstrate that screening retrovirally expressed random peptides can identify unique peptide sequences that affect specific biological functions and thereby facilitate the development of novel therapeutics.

Experimental Procedures

Screening of GFP-Fused C Terminus 20-mer Random Peptide Library

A retroviral peptide library consisting of a random 20-mer peptide with a linker (EEAAKA) fused at the C terminus of GFP in a tetracycline (tet)-regulatable (tet-off system) retroviral vector, TRA, [32] was constructed as previously reported (TRA-GFP-C20) [8]. Transfection of the library into an amphotropic retrovirus packaging cell line, ϕNX-Ampho, which produces infectious retrovirus up to 5×10^6 infectious U/ml was carried out as described previously [33]. Tetracycline-regulatable transactivator (tTA)-expressing lung tumor cells, A549.tTA [32], were spin infected with the supernatant from ϕNX-Ampho cells at 2500 rpm for 1.5 hr at 32°C and were incubated for 12 hr at 32°C. Cells were then incubated at 37°C for 24 hr to allow integration and expression of the retrovirus. Cells were then stained with a cell tracker dye, 5 μM DiI (D-282, Molecular Probes, Inc., Eugene, OR), for monitoring proliferation as per manufacturer's recommendation. After infection with the retrovirus encoding diphtheria toxin α chain, cells were sorted for DiI bright and GFP-positive cells with a fluorescence-activated high-speed cell sorter, MoFlo (Dako Cytomation, Inc., Fort Collins, CO) in order to enrich for nondividing cells. After another round of diphtheria toxin selection and cell sorting, A549.tTA cells were plated into 384-well plates for single-cell cloning in 30 μl media containing 10 μg/ml of a tetracycline analog, doxycycline (Dox). Colonies were picked and analyzed for growth in both Dox-containing and Dox-free media. Clones that showed both reduction of cell growth in absence of Dox and normal cell growth in presence of Dox were identified. Total RNA was extracted from these clones, and cDNAs encoding peptide library elements were rescued using RT-PCR (AMV reverse transcriptase from Promega, WI and Vent DNA polymerase from New England Biolabs, Inc., Beverly, MA). The resulting cDNAs were sequenced and religated into the TRA vector. Following infection of naïve A549.tTA cells with retrovirus encoding rescued peptides, the antiproliferative effect of the peptides were confirmed with the cell tracker assay.

Cell Tracker Assay

A549.tTA cells were infected with tet-regulatable retroviruses expressing GFP-fused peptides, stained with the cell tracker dye, DiI, and incubated at 37°C for 5 days. After the incubation, fluorescent intensity of GFP and DiI was analyzed with MoFlo. A "fold inhibition" was determined as the ratio of mean DiI fluorescent intensity for the GFP-positive population to that of the GFP-negative population.

Immunohistochemical Staining

A549.tTA cells expressing GFP-fused peptides 38 and 41 were plated on coverslips, washed with phosphate buffer saline, PBS, and fixed in 50% methanol and 50% acetone for 5 min. Cells were then washed twice with PBS, incubated with PBS containing 0.5% saponine and 0.5% BSA for 20 min. After washing cells with PBS twice, cells were incubated in PBS containing 0.5% saponine and 0.5% BSA at room temperature for 1 hr with goat anti-clathrin heavy chain antibody (Sigma) or mouse anti-vinculin antibody (Transduction laboratories), washed intensively, and then incubated with the secondary Alexa Fluor^R 546-labeled donkey anti-goat IgG antibody (Molecular Probes) or Alexa Fluor^R 546-labeled goat anti-mouse IgG antibody (Molecular Probes), and Alexa Fluor^R 488-labeled rabbit anti-GFP antibody (Molecular Probes). Both primary and secondary antibody solutions were precleared by centrifugation. After staining, the coverslips were mounted in Fluoromount-G (Fisher Scientific, Pittsburgh, PA). The samples were examined on a fluorescent microscope, Axiovert S100 (Carl Zeiss MicroImaging, Inc., Thornwood, NY), equipped with a 63 Å ~1.4 numerical aperture oil immersion objective lens and the single fluorochrome filter sets for either Texas red or fluorescein were used for visualization and recording of the images. The images were captured with a CCD camera, model C4742-95-12ER (Hamamatsu Photonics K.K., Hamamatsu, Japan)

Poly Lysine and FITC-Labeled Synthetic Peptides

Synthetic peptides fused with seven Lys (K7) via a linker (GGEEAAKA) at the N terminus (K7_40, K7_40M, K7_41, K7_41M, K7_41_FITC, and K7_41M_FITC) were purchased from New England Peptide Inc., Fitchburg, MA and American Peptide Company, Sunnyvale, CA.

High-Content Imaging of Antiproliferative Peptides

A549 cells (American Type Culture Collection [ATCC], Manassas, VA) were plated at 2000 cells/well in 96-well plates with F12K media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and allowed to grow for 24 hr. Peptide dissolved in media was added to the cells to a final concentration ranging from 0.8 to 25 μM in triplicate. Following incubation with peptide for 24 hr, cells were fixed with 3.7% formaldehyde for 30 min, rinsed in PBS, and stained with DAPI (Molecular Probes) to label nuclear DNA. Digital images of the labeled nuclei in each well were obtained using a Zeiss Axiovert microscope, UV filter set, and Photometrics camera. Images were analyzed using the Image Pro software package to count the number of nuclei.

Acknowledgments

We gratefully thank Dr. Nigel Bunnett, University of California, San Francisco, for helping our confocal image analysis, and Monette Aujay and Mel Fox for DNA sequencing support. We gratefully acknowledge Carolyn Sousa for critical reading of the manuscript and discussion.

Received: June 16, 2003
Revised: August 8, 2003
Accepted: August 11, 2003
Published: October 17, 2003

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