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Cellular Interacting Proteins of Functional Screen-Derived Antiproliferative and Cytotoxic Peptides Discovered Using Shotgun Peptide Sequencing

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Summary

Three nuclear-excluded antiproliferative peptides discovered in an intracellular peptide library screen contained variants of a leucine-rich nuclear exclusion motif. When tagged with N-terminal lys_7, two peptides, but not quadruple alanine motif mutants, killed A549 cells. Cellular proteins bound to N-biotinylated analogs, but not to biotinylated inactive mutants, included a set of proteins involved in nucleocytoplasmic transport, including exportin-1, importin β subunits, importin α, nucleoporin p62, and the GTPase ran. The peptides, but not mutants, also bound other protein sets including tubulin and actin, elongation factors, mRNA-associated splicing factors, and proteins associated with DNA replication such as PCNA, annexin II, and calpactin. Many extracted proteins are linked to nucleocytoplasmic transport, and some are involved in cell cycle regulation. These peptides may act by disrupting nucleocytoplasmic transport of proteins important for cell growth.

Introduction

Cellular processes are tightly regulated by the activation and deactivation of series of proteins that constitute signaling pathways. An approach to discover novel elements of these pathways in the context of their functional biology will increase understanding of the pathways and of the phenotypes being studied, and may allow discovery of novel drug targets. Cellular screens of random peptide libraries have been used to discover novel peptides or proteins with specific cellular phenotypic effects in yeast [1–3] or in human cells following retroviral delivery of peptide libraries [4–7]. These screens do not require prior knowledge of protein interactions or signaling pathways involved in phenotypic changes, and thus novel pathway members or interactions may be discovered. Peptide library members may bind cellular macromolecules as part of their phenotypic effect. It is often not obvious how these peptides or proteins cause their phenotypic effects within the cell. Besides detailed analysis of the exact phenotypic effects, an important step toward understanding function involves determination of their cellular binding partners. Identification of these interacting partners may elicit hypotheses of mechanism and may reveal potential drug targets for treating the disease.

A screen was undertaken for antiproliferative peptides, involving retroviral delivery of a 10^9 member random 20-mer peptide library (fused to the C terminus of GFP [4]) to a similar number of A549 cells and selection of nondividing cells by celltracker dye fluorescence and resistance to infection with a retrovirus producing diphtheria toxin alpha [5]. Here we examine common sequence features of three of the peptide hits resulting from this screen; the results provide an explanation for their nuclear exclusion. Using high-content imaging, we examine dose-dependent phenotypic properties of synthetic analogs of two of these peptides designed to internalize into cells, as well as mutant peptide controls. We also examine the cellular interacting partners of biotinylated synthetic analogs of three of these peptides, comparing LC/MS/MS-based shotgun peptide sequencing of tryptic digests of quadruplicate active peptide affinity extracts with inactive mutant peptide extracts. For a more comprehensive analysis of the cellular interacting proteins, we combine the above analysis with MALDI-TOF mass spectrometry of in-gel tryptic digests of difference gel bands, and Western blotting. Finally, we examine common properties of the combined 43 interacting proteins of the three peptides. The three peptide hits bind a core of similar proteins, and thus could act by similar mechanisms, as well as proteins unique for each peptide, suggesting important differences. Based on the properties of the interacting proteins, hypotheses to explain peptide-based growth inhibition are discussed.

Results

Peptide Sequences May Explain Peptide Nuclear Exclusion

The peptide hits were derived from a random peptide library; by BLAST analysis, none of the sequences are identical to fragments from a known protein. However, since all of the peptides are at least partly nuclear excluded, their sequences were aligned with those of nuclear excluded proteins with leucine-rich nuclear exclusion motifs (Table 1). HIV-1 Rev or HTLV-1 Rex has a leucine-rich motif and is recognized by a complex including exportin-1 for transport out of the nucleus [8–10]. The leucine-rich motifs are characterized by three leucines separated by 2–3 other residues, and a fourth leucine separated by a single residue. The leucines are sometimes replaced by other hydrophobic residues

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Table 1. Sequence Alignments of Three Antiproliferative Peptides with Nuclear Exclusion Motifs

Peptide	Sequence[a]	Comment
35	RLRRICSGILL IRRI LG IFV	
40	RWDPTRLLR FRF LR MLVRRSRPVR	
41	GRGCIFRWRRG LRGM MR LFK	
HIV-1 Rev	LPP LER LT LD	leu-rich motif
HTLV1 Rex	LSAQ LYSS LS LD	leu-rich motif
PKI	LALK LAG LD IN	leu-rich but I substutes for L
HDM-2	LSLS FDES LA LC	leu-rich but F substitutes for L
General motif	X X X X	X is a hydrophobic residue

[a] The sequences shown are those of the random peptide library member; all were fused to the GFP C terminus via a spacer sequence, EEAAKA.

such as phe or ile. If other hydrophobic residues such as met and trp are allowed to replace some leucines, peptides 35, 40, and 41 have sequences that fit this more general motif.

Synthetic Peptide Analogs with Fused Internalization Domains Cause a Dose-Dependent Abnormal Nuclear Morphology and Cell Death

Polybasic peptides can efficiently internalize fused macromolecules into cells [11–13]. The effects of N-terminal lys_7-tagged peptides 40 and 41 and their mutant controls on A549 cells were examined using high-content imaging fluorescence microscopy [14]. Figure 1A shows examples of A549 cell nuclei that have been stained with the DNA binding dye DAPI following 48 hr exposure to 50 μM peptide. Cells exposed to the tetra-alanine mutants (Figures 1A1 and 1A3) are similar to untreated control cells and exhibit a range of nuclear morphologies characteristic of A549 cell populations, containing cells at metaphase (open arrow) and anaphase (solid arrow). In contrast, cells exposed to peptides 40 and 41 (Figures 1A2 and 1A4) exhibit a significant cell loss. Many remaining nuclei have an abnormal nuclear morphology (arrow, Figure 1A2), being small and rounded and staining intensely for DAPI, suggesting a high degree of chromatin condensation. Cells treated with peptide 41 exhibit a higher fraction of doubled cells (4 of 30 cells) than peptide 41 mutant (ca. 4 of 150 cells). These cells appear similar to those labeled as mitotic anaphase cells in Figure 1A1 but may not be identical. This suggests this may be a specific effect of this peptide in cells that are not yet dead. Figure 1B shows the results of the peptide dose dependence of the number of cell nuclei remaining in a 96 well plate, originally seeded with 2000 cells, after 48 hr exposure to peptides added to the cell culture medium. Tetra-alanine mutants of both peptides showed no dose-dependent effect up to 50 μM levels. Both peptides 40 and 41 exhibited a dose-dependent decrease in the number of cell nuclei observed, with a midpoint concentration of ca. 10 μM. Figure 1C shows the dose dependence of the appearance of abnormal nuclear morphology for peptides 40 and 41 after 48 hr exposure to A549 cells. The control alanine mutant peptides had no observable effect, while both lys_7-tagged peptides were associated with a dose-dependent increase in abnormal nuclear morphology with midpoints in the 10–20 μM range. Lys_7-tagged peptides 40 and 41, but not tagged alanine mutant control peptides, thus can cause a dose-dependent cell death and a dose-dependent change in the appearance of the nuclei of the remaining cells.

In-Gel Tryptic Digests of Difference 1D Gel Bands and MALDI-TOF Mass Spectrometry Identify Binding Partners of Three Peptides

To further examine the action of these peptides in cells, peptides 35, 40, and 41 were synthesized with the sequence biotin-GMDELYKEEAAKA added to their N terminus. The residues MDELYK were from the C terminus of GFP; the residues EEAAKA were spacer residues between the GFP β-can structure and the peptide sequence selected in the functional screen. The GFP β-can was thus replaced with a biotin. Inactive alanine mutant peptides, with all four of the bold residues shown in Table 1 mutated to alanine, were used as controls. A549 cell affinity extracts using the active peptide sequences were compared to control extracts using one-dimensional silver stained gels. Figure 2A shows the results of this comparison for peptides 41, 40, and 35. Difference bands were identified using MALDI-TOF mass spectrometry and matching of the masses of the in-gel digest tryptic peptides to predicted peptide masses for individual proteins using the programs Mascot [15] or Profound [16]. The resulting identifications, with the number of matching tryptic peptides, are included in the middle columns of Table 2 for peptide 41. Each identified protein had a predicted molecular mass within 10% of the observed mass. Seven interacting proteins, including three subunits of importin-β, β-tubulin, BiP, annexin II, and calpactin-1/S100, were identified for peptide 41. Each was identified by 10–20 matching tryptic peptides.

For peptide 40, six binding partners were identified, including importin-β subunits 1 and 3, β-tubulin, elongation factor tu, a Kruppel-type zinc finger, and an ATP/ADP carrier protein (Table 2). All were identified by at least 11 peptides except importin-β 3, which was identified by 8 peptides. All identified proteins were within 10% of the observed mass except for the zinc finger protein, which was within 22%. For peptide 35, five interacting partners were identified, including importin-β 1, β-tubulin, actin, restin, and KIAA0052. Thus these three functional peptides appear to share interactions with a number of proteins, as well as bind to proteins unique for each peptide.

LC/MS/MS-Based Shotgun Peptide Sequencing and Western Blotting Identify Additional Interacting Proteins

To find additional interacting proteins, quadruplicate affinity extracts using each biotinylated peptide were sep-

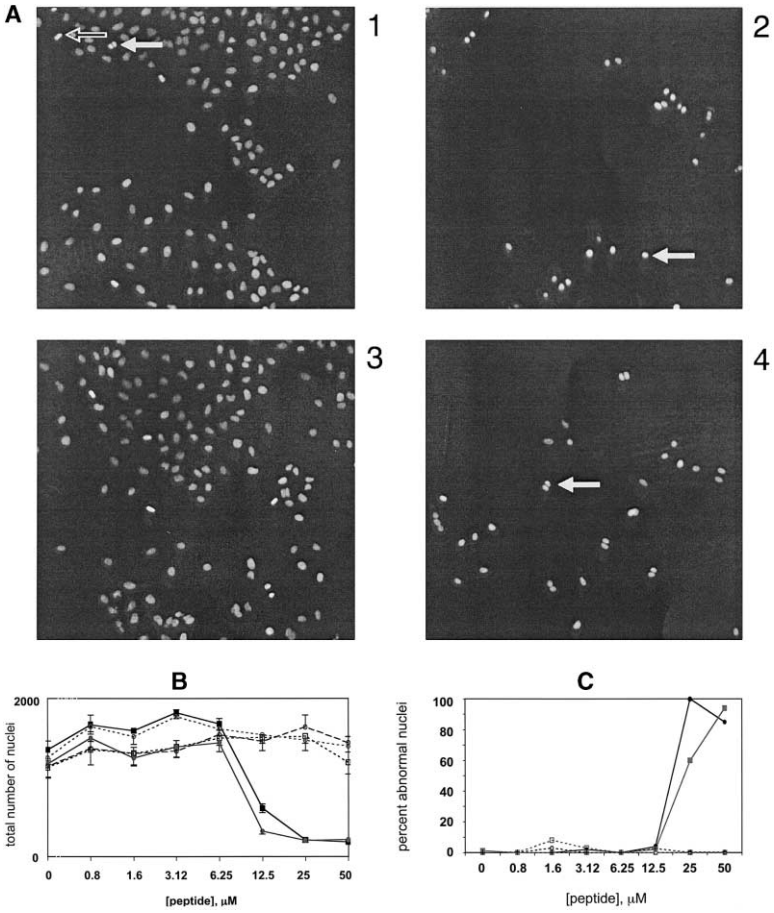

Figure 1. Phenotype of Synthetic Peptide Analogs in A549 Cells

(A) Fluorescence microscopy of A549 cells treated with lys_7-peptides and stained with the DNA binding dye DAPI. Individual panels are for cells examined 48 hr after treatment with 50 μM of mutant peptide 40 (A1), peptide 40 (A2), mutant peptide 41 (A3), and peptide 41 (A4). Cells exposed to lys_7-tetra-alanine mutant peptides have a normal nuclear morphology, while cells exposed to lys_7-active peptides exhibit significant cell loss and small, rounded, intensely staining nuclei consistent with chromatin condensation. Mitotic cells are labeled with arrows; open arrow, metaphase cells; solid arrows, anaphase cells (A1 and A4). Cells remaining after treatment with peptide 41 have a higher fraction of mitotic cells (4 of 30 cells) than with mutant peptide 41 (4 of ca. 150 cells). The solid arrow in (A2) indicates a rounded cell with abnormal nuclear morphology.

(B) Effect of lys_7-peptides on A549 lung carcinoma cells. Lys_7-peptides 40, 41, or inactive tetra-alanine mutants of each were added to the culture medium of 2000 cells in a well at increasing doses, and a Cellomics high-content screening system was used to observe cells 48 hr after peptide addition. Filled circles represent the active peptide 41 sequence; filled squares represent the active peptide 40 sequence; open circles with a finely dashed line represent inactive mutant peptide 41; open squares represent inactive mutant peptide 40; open circles with a dashed line represent untreated cells. Values were averages of triplicate measurements ± one standard deviation. Peptides 40 and 41 caused a dose-dependent decrease in the number of observed cell nuclei to ca. 10% of original values. The mechanism of cell death is not defined.

(C) Effect of peptides causing abnormal nuclear morphology of A549 cells after a 48 hr exposure to varying doses of peptides. Symbols are as in (B) above. Peptides 40 and 41 both cause a dose-dependent increase in cells with abnormal nuclear morphology not seen with their corresponding mutant peptides.

arately digested with lys-C endoprotease and then with trypsin. Quadruplicate extracts using the control biotinylated inactive peptides, each of which had the four nuclear exclusion motif residues (Table 1) mutated to alanine, were digested in the same fashion. The entire affinity extract digests were then chromatographed on a microcapillary C18 reversed-phase HPLC column, the mass of each peptide was measured, and the peptide was then fragmented in an ion trap mass spectrometer. LC/MS/MS data from the active sequences were then compared to data from the control affinity extracts using the Oracle database MEDUSA [17]. Proteins unique to the active peptide affinity extracts, with at least one good sequenced peptide using published criteria [18, 19], and present in at least two of four extracts were identified. The individual tryptic peptides used for the identifications were independently BLASTed to check for ambiguity in identifications. The numbers of good peptides, and total peptides, used in the identifications are listed in Table 3.

For peptide 41, this mass spectrometry data revealed the presence of two types of β-tubulin, α-tubulin, actin, as well as myosin. Importin-β 2, annexin II, and calpactin were also found. Two elongation factors, two hnRNP proteins, a protein phosphatase, a tRNA synthetase, and a DNA binding protein were discovered. For peptide 40, α and β-tubulin and α-actin were present, as were a splicing factor and hnRNP protein, two elongation factors, two proteins associated with DNA replication, proliferating cell nuclear antigen (PCNA) and a DNA ligase-like protein, two reticulocalbins, calumenin, nucleobindin, two unknown proteins, and several enzymes. For peptide 35, fewer proteins were reproducibly present in the affinity extracts. These included α- and β-tubulin and DNA-directed RNA polymerase IID.

To confirm the presence of a number of these proteins, Western blotting was done on the affinity extracts when antibodies were available. Blots of whole-cell lysates were included, as well as positive control proteins when available. Blotting was not done for tubulin, actin, and myosin, since the identifications from LC/MS/MS data involved multiple sequenced peptides. Figure 2B shows the results of Western blots for proteins listed in Table 2. The presence of PCNA was confirmed in affinity extracts of all three active peptides but not in the extracts using the mutant peptides. Calpactin/S-100 was present in two extracts, but not in controls, and more was present in a third extract than in the control. Annexin II, BiP, and elongation factor tu were differentially present in the peptide 41 extract, but were not observable in the

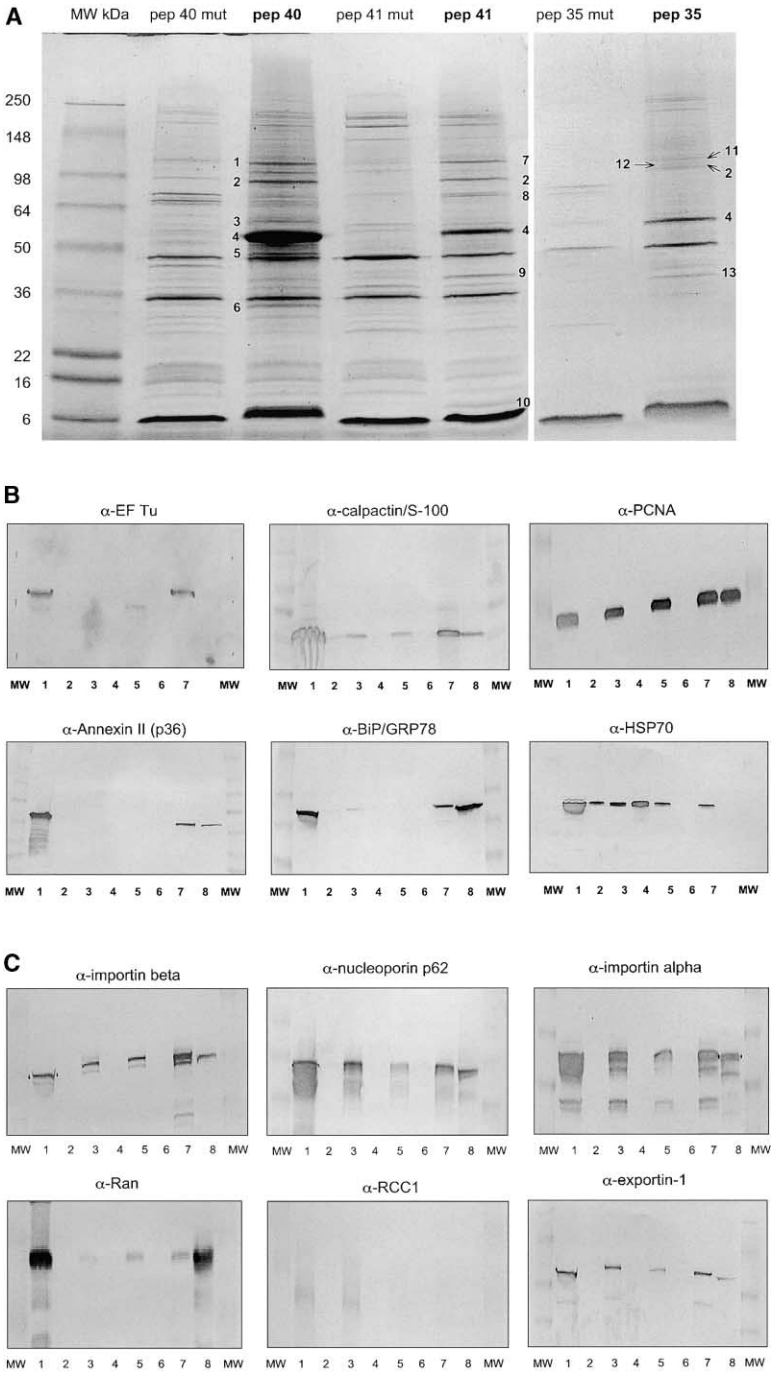

Figure 2. Biotin-Peptide Affinity Extracts Examined by 1D SDS-PAGE and Western Blotting

(A) Lysates of ca. 10^8 A549 cells, treated with a protease inhibitor cocktail, were affinity extracted with biotin-GMDELYKEEAAKA-peptide 40, 41, and 35 in separate experiments. Peptides with the four leucine-rich motif residues mutated to alanine, which were inactive, were used as controls. Bands present at higher levels in the active peptide affinity extract compared to the mutant peptide extract are labeled. Each peptide extracted 5–6 noticeable bands, some of which were common to more than one peptide extract. The largest, band 4, was identified as β-tubulin by MALDI-TOF mass spectrometry; band 2 was identified as an importin β subunit.

(B) Western blots of proteins present in A549 cell affinity extracts of peptides 35, 40, and 41. Adjacent lanes consist of affinity extracts using biotinylated peptides and biotinylated inactive mutant peptides. Lanes 1 and 8: Western blots of cell lysate and positive control proteins when available; lanes 3, 5, and 7: peptides 35, 40, and 41; lanes 2, 4, and 6: inactive mutant peptides 35, 40, and 41. Western blotting confirmed some proteins found by mass spectrometry. Elongation factor tu was confirmed in extracts of peptide 41 and perhaps peptide 40; calpactin and PCNA are present in all three peptide extracts. Annexin II, HSP70, and the chaperonin BiP are differentially present in the peptide 41 extract but not the mutant controls. HSP 70 is present in all other peptide extracts and controls.

(C) Western blot of proteins associated with the nuclear pore complex or nucleocytoplasmic transport. Importin β (containing 2–3 bands) was differentially present in all three peptide extracts, as was nucleoporin p62, importin α, ran, and exportin 1. RCC1 was present in the peptide 35 extract but missing from the controls.

other extracts. Elongation factor tu and heat shock protein 70 were differentially present in the peptide 41 extract, and a lower band was differentially present in the peptide 40 extract. Due to the presence of importin β subunits indicated by combined mass spectrometry data, antibodies binding to other nuclear pore complex proteins were also tested in Western blots. Figure 2C shows data from Western blots for nuclear pore complex proteins. Importin-β, importin-α, nucleoporin p62, the GTPase ran, and exportin-1 are present in all three peptide affinity extracts but not in control extracts. Up to three bands were visible for importin-β, which may reflect the presence of more than one subunit with differing masses, consistent with the MALDI-TOF mass spectrometry data for peptides 40 and 41. RCC1, a nuclear GDP-GTP exchange factor promoting the GTP-bound form of ran GTPAse, was present in the peptide 35 extract but below the limit of detection in extracts of the other peptides. Importin α and nucleoporin may be partially degraded in these preparations as indicated by the presence of multiple bands.

A summary of identified interacting proteins for each peptide, obtained from the three different methods, is contained in Table 3. All of the peptides differentially

Table 2. Combined Identification of Peptide-Interacting Proteins

LC/MS/MS/MEDUSA	# Peptides[a]	1D Gel, MALDI-TOF MS	Gel Band	# Peptides	Western Blot
Peptide 41					
β Tubulin 2	3, 3	β tubulin	4	13	
β Tubulin 5	3, 4				
α Tubulin	6, 6				importin α
Myosin heavy chain	2, 3				nucleoporin p62
α, β, or γ-actin	2, 2				exportin-1
(Importin β2)	0, 1	importin β1	2	16	importin β
		importin β3	7	14	Ran
		importin β7	7	13	
Annexin II	2, 2	annexin II	9	13	annexin II
Calpactin/S-100	0, 3	calpactin I/S100	10	10	calpactin I/S100
Elongation factor tu	2, 3				elongation factor tu
Elongation factor 1α 1	4, 4				
Glycyl-tRNA synthetase	3, 3				
hnRNP-U	1, 2				HSP 70
hnRNP-F	1, 1				hnRNP-F
Protein phosphatase 1G/2Cγ	1, 2				PCNA
DNA-binding protein A	2, 2	BiP/GRP-78	8	20	BiP/GRP-78
Peptide 40					
β tubulin	7, 8	β tubulin	4	13	
α tubulin	9, 9				
α-actin	2, 3				nucleoporin p62
		importin β1	2	16	importin β
		importin β3	1	8	exportin-1
		Kruppel Zn finger	3	11	importin α
SF2p32 splicing factor	6, 7				Ran
hnRNP-M	2, 3				hnRNP-F
Elongation factor tu	5, 6	elongation factor tu	5	12	elongation factor tu
Elongation factor 1α 1	3, 6				
Ribonuclease inhibitor	3, 3				
DNA ligase-like protein	1, 1				
PCNA	1, 1				PCNA
Nucleobindin 2	2, 2				calpactin
Reticulocalbin 1	5, 5				
Reticulocalbin 2	1, 1				
Calumenin	4, 5				
Paraoxonase 2	2, 2	ATP/ADP carrier protein	6	15	
ATP synthase α,β subunits	2, 2-β				
	2, 2-α				
Pyrroline-5-carboxylate reductase	3, 3				
Peptide 35					
β tubulin	4, 4	β tubulin	4	13*	Ran
α tubulin	4, 6	restin	12	12	importin α
		importin β1	2	16	importin β
					exportin-1
		KIAA0052	11	10	nucleoporin p62
(DNA-directed DNA polymerase ε)	0, 1				RCC1
(DNA-directed RNA polymerase IID	1, 1				PCNA

[a] The pairs of numbers in this column represent the number of good peptides identifying a protein, and total matched peptides for that protein.

bind cytoskeletal proteins. These include tubulin, actin and myosin, and the microtubule associated protein restin [20], which may regulate microtubule stability [21] and is involved in the binding of endosomes to microtubules. Peptide 40 binds significantly more tubulin than peptides 35 or 41, suggesting that polymerized tubulin may interact with this peptide. Other sets of interactors are discussed below.

Discussion

We have examined the interacting partners of three peptides that are antiproliferative when produced within A549 cells fused to the C terminus of GFP. Two were N-terminally fused to a lys_7 sequence and, when added outside cells, produced a dose-dependent cell death, abnormal nuclear morphology for the remaining cells, and in the case of peptide 41, an increased fraction of cells with doubled nuclei. This is a specific effect linked to a nuclear exclusion motif in these peptides, since mutations of four motif residues to alanine abolished this activity. Peptides 35, 40, and 41 bind importin β, which associates with annulate lamellae in the cytoplasm [22], which could partly explain the cytoplasmic localization of one or more of these peptides.

We have used differential affinity extractions, compar-

Table 3. Sets of Protein Interactors of Three Functional Peptides as Identified by Affinity Mass Spectrometry

Peptide	Cytoskeletal	Nucleocytoplasmic Transport	Protein Synthesis; Pre-mRNA Splicing	ER or Golgi Proteins	DNA, Replication Related
40	tubulin	importin β	EF-tu	reticulocalbin 1, 2	DNA ligase-like
	actin	exportin 1	EIF1α1, 2	calumenin	PCNA
		nucleoporin p62	hnRNP M	nucleobindin 2	Kruppel Zn finger
		importin α	hnRNP F		calpactin 1
		ran	SF2p32 pre-mRNA splicing factor		
			RNase inhibitor		
41	tubulin	same as above	EF-tu		PCNA
	myosin		EIF 1α1, 2, β1-like		DNA-binding protein A
	actin		hnRNP-U, F		calpactin
			protein phosphatase 1G		annexin II
35	tubulin	same as above	DNA-directed RNA polymerase IID		PCNA
	actin	RCC1			DNA-directed DNA polymerase ε
	restin				calpactin

ing biotinylated active peptides with tetra-alanine mutants, to identify interacting proteins that may be involved in the function of these peptides. Combining MALDI-TOF MS, capillary LC/MS/MS, and Western blotting, a total of 13, 28, and 24 interacting proteins were obtained for peptides 35, 40, and 41, respectively. Figure

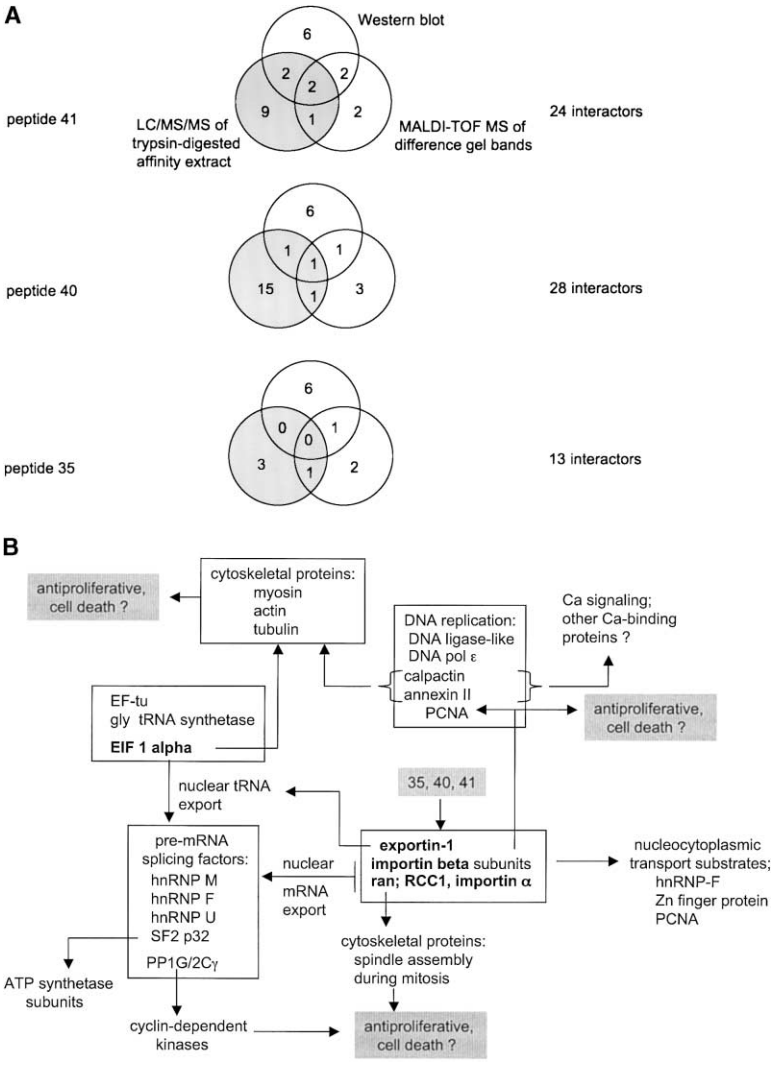

Figure 3. Venn Diagrams Showing Peptide-Interacting Proteins Defined by Different Methods and Their Functional Interconnections and Link to Nucleocytoplasmic Transport

(A) LC/MS/MS analysis of trypsin-digested entire affinity extracts overall identified the most interacting proteins and the most unique proteins. Western blotting, and MALDI-TOF mass spectrometry of in gel digests of silver stained difference gel bands, also identified unique proteins. Combination of these methods results in the most comprehensive analysis of cellular interacting proteins.

(B) The three nuclear excluded peptides extracted five main sets of interacting proteins, indicated in the boxes. These sets of proteins have linked functions as indicated; one common element is interactions with nucleocytoplasmic transport system proteins indicated in the central box, suggesting that the primary effect of the peptides is to bind one or more of these elements and disrupt nucleocytoplasmic transport. See Discussion for more details.

3A shows Venn diagrams of the results of these different methods of identifying interacting proteins. Western blotting confirmation of all proteins was not attempted, thus more proteins could be identified by this method. Each method contributes unique interactors; proteins common to more than one method help validate the methodologies. A comprehensive examination of cellular interactors should thus include all three methods. Additional interacting proteins may be missed if the four mutated residues in the control peptides are not important for their binding to the peptide; these differential extracts may thus select proteins binding the leucine-rich NES motif. These peptides bind common sets of proteins, but also unique proteins (Table 3). Peptide 35 may weakly bind more proteins also extracted by peptides 40 and 41, as a number of these proteins (elongation factor tu, reticulocalbin 1 and 2, calumenin, DNA binding protein A, hnRNP-F, splicing factor 2 p32, myosin heavy chain, actin, importin β 1, and ATP synthase) were present in one of four LC/MS/MS-examined affinity extracts but were absent from the quadruplicate controls.

The individual peptides bind common sets of proteins, suggesting similarities in their mechanism of action. They also bind multiple functional classes of proteins and may thus be present in more than one complex. The range of intensities of the difference silver stained bands are consistent with formation of different complexes present at different levels. Individual peptides also have unique interacting proteins. There may thus be more than one mechanism for the antiproliferative activity of the peptides. Additional sets of bound proteins are functionally linked to the nucleocytoplasmic transport system and to each other (Figure 3B). The extracted proteins may thus represent a snapshot of proteins linked both to the nucleocytoplasmic transport system and to antiproliferative function in these cells.

One set of extracted proteins consists of nucleocytoplasmic transport proteins including exportin-1, several different subunits of importin β, importin α, nucleoporin p62, the GTPase ran, and for peptide 35, the ran guanine nucleotide exchange factor RCC1. The isolation of these proteins is consistent with the observed nuclear exclusion of these peptides and the presence of a motif very similar to the leucine-rich nuclear exclusion motif, validating the identification of interacting proteins by this approach. Peptide-mediated antiproliferative activity or cell death could thus involve disregulation of the nucleocytoplasmic transport system. Aberrant nucleocytoplasmic transport of transcription factors has been linked to apoptosis in retinal cells [23]. Exportin-1 is involved in the nuclear export of proteins with the leucine-rich NES motif [24, 25] and interacts with and requires the GTPase ran [26], also isolated here. Nucleoporin p62, isolated with all three peptides, binds the importin α and β heterodimer, along with ran, during nuclear import [27, 28]. Peptides 40 and 41 extract multiple subunits of importin β. Individual complexes involving importin β are not currently thought to contain multiple different importin β subunits, and thus these peptides could bind independently to each of several different importin β subunits. Peptide 41 binds hnRNP-F and importin β-2, a known importer-substrate pair [10]. Peptide 40 binds a Kruppel type zinc finger; members of this family are nuclear localized due to basic residues within the zinc finger common to all family members [29]. These peptides thus also affinity extract importer-substrate pairs. Besides containing a sequence similar to the leucine-rich nuclear export motif, peptides 35, 40, and 41 are highly basic, containing 5, 9, and 7 basic residues, respectively. None of the peptides contain a classical or bipartite nuclear localization motif thought to allow binding to importin α [30]. However the basic residue compositions of 25%, 38%, and 35% are in the range of those of partially (30%) and fully nuclear-localized peptides (36%) derived from the same GFP C-terminally fused random 20-mer peptide library [4], and these peptides do bind importins α and β. They may act by binding both the nuclear export and import apparatus and compromising nucleocytoplasmic transport.

Since all three peptides are nuclear excluded, they could sequester nuclear proteins critical for cell proliferation outside the nucleus. One such protein is PCNA, which is critical for initiation of DNA synthesis and for DNA replication and repair [31]. Antisense oligonucleotides to PCNA block cell proliferation [32, 33] and prevent entry of G1 cells into S phase [34]. The ran mutants Q69L and T24N block PCNA accumulation in the nucleus and block DNA replication [35]. A second candidate is the GTPase ran, which is critical for mitosis, regeneration of the nuclear envelope and nuclear assembly, nucleocytoplasmic transport of a variety of macromolecules, DNA synthesis and replication, and RNA processing and export [36–38]. A third candidate is the nuclear guanine nucleotide exchange factor RCC1, which is essential for nucleocytoplasmic transport [39]. Loss of RCC1 induces G1 arrest and defects in nuclear transport [40] and disruption of nuclear assembly and DNA replication [36].

A second set of extracted proteins includes protein synthesis-related proteins: elongation factor tu, elongation initiation factors 1α 1 and 2, and glycine tRNA synthetase. Besides binding aminoacyl-tRNA and ribosomes, elongation factor 1α binds actin filaments and microtubules [41] in a calcium/calmodulin-regulated fashion [42] and severs stable microtubules during the cell cycle [43].

Peptides 41 and 40 also bind a third set of proteins, a total of five different pre-mRNA splicing factors. These include the hnRNPs M [44], F, and U [45], the splicing factor 2-associated protein p32, and protein phosphatase 1G/2Cγ [46]. hnRNP F is a pre-mRNA splicing factor [47] imported into the nucleus using transportin/importin β 2 [24, 48], which binds RNA polymerase II [49]. Importin β and RNA polymerase II are both present in the peptide 35 extract. Protein phosphatase 1G/2Cγ is physically and functionally associated with pre-mRNA splicing [46]. Type 2C protein phosphatases also dephosphorylate cyclin-dependent kinases [50]. Protein serine/threonine phosphatase 1G/2Cγ expression blocks DNA synthesis and results in accumulation of cells in early G1 and S phases of the cell cycle [51]. The splicing factor 2-associated protein p32 may regulate RNA splicing by blocking splicing factor 2-RNA binding and phosphorylation [52]. It is also found in the mitochondrial matrix, where it is hypothesized to play a role in oxidative phosphorylation [53]. Interaction of this protein with oxidative phosphorylation proteins could explain the presence of

ATP synthetase subunits α and β in the peptide 40 affinity extract.

The second and third sets of extracted proteins may be related. Nuclear export of mRNA is coupled to pre-mRNA splicing [54]. mRNA splicing, the transcriptional apparatus, and transcriptional elongation are coupled in eukaryotic cells [49, 55] through an RNA polymerase-splicing protein complex [56–57]. Nuclear tRNA export depends on exportin, elongation factor 1 α, and tRNA aminoacylation, coupling the protein translation, and nuclear tRNA processing and transport machineries [58]. Ran and importin β can function as nuclear mRNA export factors [59]. Peptide binding to nucleocytoplasmic transport proteins may thus disrupt tRNA export, mRNA splicing, protein synthesis, or microtubule function during the cell cycle.

A fourth set of interactors includes proteins associated with DNA replication, binding, or cell proliferation. A DNA ligase-like protein may join breaks in duplex DNA during replication, repair, or recombination. PCNA forms a trimeric ring at the DNA replication fork which is the core of a complex of enzymes involved in DNA replication [60] including a protein critical for replication, DNA polymerase epsilon [31], which was affinity extracted with peptide 35. PCNA is thus a critical protein for DNA replication and cell cycle control [60]. Peptide 41 affinity extracts annexin II and calpactin as well as α and β tubulin, actin, myosin, and PCNA. Calpactin is an EF-hand calcium binding protein that forms a cytoskeletal-bound heterotetramer with annexin II [61]. This could explain the presence of tubulin, actin, and myosin in the peptide 41 affinity extract. Annexin II is part of the primer recognition complex [62, 63] and is important for DNA synthesis and cell proliferation. Immunodepletion of annexin II blocks DNA replication [64]. Peptide 41 could thus block proliferation by interfering with DNA replication. Calpactin and annexin II are involved in calcium signaling [65, 66], as are the endoplasmic reticulum or Golgi-resident multiple EF-hand proteins reticulocalbin, the cytosol and Golgi DNA binding protein nucleobindin/calnuc [67], and calumenin, all isolated here by peptide 40.

All three peptides bind importin α, importin β, and ran, and peptide 35 also binds RCC1. Ran regulates microtubule polymerization during mitosis [68–70]. These four proteins are involved in different ways in spindle assembly during mitosis [71–73]. It is possible that these peptides disregulate this process, resulting in the observed accumulation of mitotic nuclei for peptide 41.

It is difficult to compare our results with colocalization studies presented in the preceding paper [5]. Each peptide affinity extracts multiple different proteins, making a prediction of colocalization difficult. Further, the localization of the GFP-fused peptides can be complex. GFP-peptide 41 localizes to cytoplasmic structures and to the plasma membrane in some A549 cells, but in others it exhibits a perinuclear localization similar to the nuclear membrane, and in others it localizes to the membrane between attached cells. This heterogeneity in localization may make it challenging to address the mechanism of action of this peptide using localization as a tool.

The three peptides examined here have nuclear exclusion motifs. These peptides bind a core of similar proteins, have elements of similarity in their sequences, and may block or disregulate a common pathway important to proliferation, such as nucleocytoplasmic transport. Disregulation could result in functional cellular changes in a number of areas, discussed above, that could compromise cell proliferation or cause cell death. The three nuclear excluded peptides have a more complex motif than a leucine-rich repeat, which could explain the frequency of these hits of ca. 1 in 10^8 in the antiproliferative screen. The leucine-rich NES motif observed here requires L, I, F, W, or M at each of four defined positions; this grouping occurs at a frequency of $[5/20]^4$. All three peptides have an arg after the second leu of this motif, and a hydrophobic residue before the third leu and after the last leu, which occur at frequencies of 1/20, $\sim$5/20, and $\sim$5/20. If the residues before and after the first leu, and after the third leu, are required to be R or G, R or L, and R or G, respectively, the frequency of occurrence of this entire set of residues is ca. $1/10^8$, similar to the frequency of these peptide hits.

Significance

Peptide or protein hits from screens of intracellular peptide libraries have been discovered that affect the yeast pheromone signaling pathway [1], transcriptional silencing or the spindle checkpoint [3], cell cycle arrest [2], germline epsilon promoter transcription [7], and that mislocalize GFP in cells [4] or that are antiproliferative. Such screens are thus broadly applicable to biological problems. To elaborate the cellular function of peptides resulting from one of these screens, we have used peptide analogs as affinity reagents to examine their cellular interacting proteins. Shotgun peptide sequencing and other methods have identified a number of proteins extracted by all three peptides as well as unique proteins for each peptide. As with other recent affinity extractions with functional baits [17, 74–78], multiple functional sets of proteins were identified. Here these proteins belong to a protein network with interrelated functions, all with linkage to the nucleocytoplasmic transport system. They connect peptide nuclear exclusion and peptide function, giving a snapshot of proteins linked to the antiproliferative xz properties of these peptides. Combining a mass spectrometry examination of affinity extracts and Western blotting can thus be a powerful approach to examining the cellular functional and interaction neighborhood of novel molecular entities, and can give new insight into the protein machinery associated with interesting cellular phenotypes. Using known or potential drug targets as baits in human cells, the protein interaction neighborhood of the drug targets may also be examined, giving more information on target function and possibly allowing discovery of additional functionally linked drug targets. Using affinity-tagged small molecule drugs or drug candidates as baits, cellular targets for these drugs may be elucidated by examining affinity extracts with and without competition from the unlabeled drug. This methodology may thus have broad applicability for the discovery of drug targets in the pharmaceutical industry.

Experimental Procedures

Affinity Extractions and Gel Electrophoresis

Affinity extractions and electrophoresis were carried out mainly as described [17]. Cultured human A549 lung cancer cells (10^8 for each affinity extraction) were pelleted, washed twice in phosphate-buffered saline, and suspended in 5 ml of 4°C 2% Triton X-100 containing a cocktail of protease inhibitors (Complete Tablets, Boehringer Mannheim, Germany), 20 mM Tris buffer (pH 7.2), 0.15 M sodium chloride, and 1 mM ethylenediamine tetraacetic acid (EDTA). After homogenization, the cells were centrifuged at 14000 × g for 20 min at 4°C, and the supernatant was cleared of proteins that may bind agarose beads by tumbling with 250 μl of a suspension of agarose beads (Pierce Chemical Co., Rockford, IL). Protein concentration was measured by a micro-BCA assay (Pierce Chemical Co.) and was normalized to 5 mg/ml in all the affinity extractions. After centrifugation, 1 ml of the supernatant was added to 250 μl of a slurry of agarose beads covalently attached to streptavidin (Pierce Chemical Co.) preincubated with an excess of each biotinylated peptide and washed three times in PBS. All peptides were synthesized by American Peptide Co. (Sunnyvale, CA) and had the correct mass for their sequence as measured by mass spectrometry. If free in solution, the peptide concentrations would be ca. 50 μM in cell lysates. At this concentration the lys_7 analogs of peptide 40 and 41 caused a loss of most cells in the 96 well plate. Quadruplicate extractions were carried out overnight at 4°C with gentle tumbling in 2 ml plastic Eppendorf tubes (16). Beads were then washed three times with 0.05 M Tris, 0.15 M sodium chloride, 0.1% Triton X-100 followed by two washes with 0.05 M Tris, 0.15 M sodium chloride, 1 mM EDTA. Quadruplicate control extracts under identical conditions were obtained using biotinylated inactive tetra-alanine mutant peptides at the same concentrations.

Washed beads were boiled in SDS-PAGE 2× sample buffer (Novex, San Diego, CA) containing 100 mM dithiothreitol (DTT). SDS-PAGE separation was on a Novex 4%–20% gradient Tris-glycine gel. Proteins electroblotted onto PVDF membranes (Novex) were probed with appropriate antibodies and developed using an ECL Plus enhanced chemiluminescence reagent kit followed by detection on ECL hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

All antibodies used in Western blots were from BD Biosciences-Transduction Labs (Lexington, KY) except antibodies to PCNA and elongation factor tu (Santa Cruz Biotechnology, Santa Cruz, CA).

Mass Spectrometry Analysis of Affinity Extracts

Microcapillary LC/MS/MS analysis was carried out as described [17]. Briefly, 5 μl of lys C/trypsin digests of streptavidin-bead affinity extracts were desalted offline on a C18 guard column (Vydac, Hesperia, CA) and injected onto a fused silica microcapillary HPLC column (15 cm long, 75 μm i.d.) packed with Nucleosil C18 (100 Å pore size, 5 μm particles). The capillary column was connected to a 15 μm PicoTip (New Objective Inc., Woburn, MA) as the electrospray tip through a stainless steel zero dead volume union where the electrospray voltage was applied. HPLC solutions were 5% acetonitrile, 0.1% formic acid (A) and 80% acetonitrile, 0.1% formic acid (B). The HPLC gradient was a 20 min gradient from 0% to 10% B, a 60 min gradient from 10% to 30% B, a 10 min gradient from 30% to 50% B, a 10 min gradient from 50% to 80% B, and a hold at 80% B for 10 min. Precursor ions were scanned from 350–1800 m/z in full-scan mode. The HPLC was an Ultimate capillary HPLC (LC Packings, San Francisco, CA). Mass spectrometry was carried out on a ThermoFinnigan LCQ ion trap. Some samples were run on a Micromass (Beverly, MA) QTOF-1 mass spectrometer. In-gel digest peptide extracts were analyzed on a Bruker Reflex III time-of-flight mass spectrometer at both Rigel and the Univ. of Alberta, and a Bruker AnchorChip using dihydroxybenzoic acid as a matrix (Rigel); some peptides were also fragmented using a PE-Sciex API-QSTAR pulsar at the Univ. of Alberta.

Database Searching and MEDUSA Analysis

Collected MS/MS data were analyzed using TurboSequest (ThermoFinnigan, San Jose, CA) against a human protein sequence database derived from the NCBI nonredundant database (http://www4.ncbi.nlm.nih.gov). Human immunodeficiency virus protein sequences were first removed from the human protein database by a database tool in the Xcalibur software (ThermoFinnigan). SEQUEST scores [79] were evaluated by the criteria of Washburn and colleagues [18], which includes peptides with a delta *Cn* of greater than 0.1 and Xcorr values greater than 1.9, 2.2, and 3.75 for +1, +2, and +3 ions, respectively. SEQUEST results were summarized and stored in an Oracle 8i database called MEDUSA [17]. Peptide masses obtained from MALDI-TOF experiments were analyzed using Mascot [15] or ProFound [16].

Fluorescence Microscopy Examination of A549 Cells

A549 tumor cells (American Type Culture Collection, Fairfax, VA) were plated at 2000 cells per 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 of 0.8–50 μM in triplicate. Following incubation with peptide for 24 or 48 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 taken using a Cellomics Arrayscan II, consisting of a Zeiss Axiovert microscope, UV filter set, and Photometrics camera. Images were analyzed using the Image Pro software package (Media Cybernetics, Silver Spring, MD) to count the number of nuclei and measure the lowest gray value for individual nuclei. The lowest gray value ranges encompassing all of the control population were then calculated, and the percentage of nuclei lying outside this range was plotted for each type of peptide.

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