Sequence Requirements for Synthetic Peptide-Mediated Translocation to the Nucleus

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The abilities of 18 synthetic peptides to target a carrier protein to the nucleus following microinjection into the cytoplasm of HeLa cells were determined. Eight of the sequences chosen for synthesis were based on published nuclear targeting regions as determined by gene fusion and deletion experiments. Six of these sequences were found to be effective when mimicked by a synthetic peptide and conjugated to a carrier protein. One additional peptide was based on a region of lamin L₁, a nuclear protein from *Xenopus laevis*, in which the nuclear targeting region had not been previously investigated. This peptide was also able to target a carrier protein to the nucleus. Eight other peptides which resemble the known targeting signals had little or no nuclear targeting ability. Peptides which were able to target a carrier protein to the nucleus did so within 45 min of injection into the cytoplasm. Two peptides with little or no apparent nuclear targeting ability after 45 min were examined for longer times as well. No increase in nuclear accumulation was observed between 45 min and 4 h after cytoplasmic injection. Comparison of the sequences which were effective at nuclear targeting with those that were not revealed a possible consensus sequence for peptide-mediated nuclear transport.

Proteins destined for the nucleus in eucaryotic cells enter through large pore structures distributed evenly over the nuclear envelope. Entry of small proteins through the pores is unimpeded, but proteins larger than approximately 40 kilodaltons require ATP (18, 19) as well as a specific nuclear targeting signal for transport (5; for reviews, see references 3 and 20). The precise sequence requirements of this signal are not well defined, but experiments based on several different approaches to this problem are beginning to shed light on what is necessary for entry. Deletion analysis and site-directed mutagenesis have narrowly defined the regions required for nuclear transport for a variety of proteins. Subsequent gene fusion of these regions with nonnuclear proteins generally results in translocation of the chimeric proteins to the nucleus.

An alternative to genetic manipulation has been chemical synthesis of a peptide which mimics a putative nuclear targeting region. The nuclear targeting sequence of the simian virus 40 (SV40) T antigen has been synthesized and cross-linked to a variety of nonnuclear carrier proteins, such as serum albumin, immunoglobulin G, and ferritin. These conjugates are translocated to the nucleus following microinjection into the cytoplasm of cells (6, 14). Changing one amino acid in the sequence (lysine 128 to either threonine or asparagine) yields a dramatic reduction in the degree of transport of the peptide conjugate into nuclei (6, 14). The results of these peptide "mutations" are consistent with results obtained by mutational analysis of the T antigen itself (10, 13).

We synthesized 2 SV40 T-antigen nuclear targeting peptides and 16 others to determine the general utility of peptide-mediated translocation and to define further the sequence requirements for nuclear transport. We found that some but not all of the genetically defined transport sequences could be mimicked by peptide conjugates. Although all of the peptides capable of directing nuclear transport contain a cluster of basic residues, this in itself is not

sufficient for transport. Most of the nontargeting peptides also contain either three or four basic residues clustered in a short sequence. A comparison of the amino acid sequences of nuclear targeting versus nontargeting peptides has led us to propose a consensus sequence for such peptides.

MATERIALS AND METHODS

Peptide synthesis. All of the peptides listed in Table 1 were manually synthesized on a RAMPs apparatus (Du Pont Co., Wilmington, Del.). Peptides P5 and P9 were made by using Wang resins (Bachem, Torrance, Calif.), which result in a free carboxyl at the C terminus. All other peptides were made with Rapidamide resin (Du Pont), which results in an amide at the C terminus. After cleavage from the resin and deprotection, peptides were purified by high-pressure liquid chromatography on a C-18 reverse-phase Vydac preparative column (Rainin Instrument Co., Woburn, Mass.) and analyzed for correct composition by fast atom bombardment mass spectrometry.

Peptide-protein conjugation. Synthetic peptides were conjugated to chicken serum albumin (CSA) with a bifunctional cross-linking reagent, *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce Chemical Co., Rockford, Ill.), essentially as described by Lanford et al. (14). MBS at 10 mg/ml in dimethylformamide (0.026 ml) was added to 0.2 ml of CSA (Organon Teknika, Malvern, Pa.) at 27 mg/ml in 50 mM sodium phosphate buffer, pH 7.5. After 1 h at 22°C, the reaction mixture was fractionated on a G150 Sephadex column (13.5 by 0.8 cm; Pharmacia, Inc., Piscataway, N.J.) in the same buffer. The protein eluted in a total of 0.7 ml, of which 0.2 ml was added to 3 mg of purified peptide, followed by 2 h of incubation at 22°C. The resulting conjugates were then dialyzed against phosphate-buffered saline to remove unreacted peptide. A sample was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining for an increase in size due to the addition of peptides to the protein. A more accurate estimate of the average degree of conjugation was determined by amino acid

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TABLE 1. Cellular localization of CSA conjugated to synthetic peptides

Peptide	Origin of sequence; residue no. (reference)	Peptide sequence ^a	Cellular localization ^b
P1	SV40 large T; 126–132 (10)	C G G <u>P K K R K V</u> G CONH ₂	N
P2	Polyomavirus large T; 189–196 (22)	C G Y G <u>V S R K R P R P</u> G CONH ₂	N
P3	<i>X. laevis</i> nucleoplasmin; 164–172 (1, 4)	C <u>G O A K K K K L D</u> CONH ₂	N
P4	<i>X. laevis</i> lamin L ₁ ; 409–421 (12)	C <u>V R T T K G K R K R I D V</u> COOH	N
P5	Human c-myc-encoded protein; 321–328 (24)	C G G <u>A A K R V K L D</u> CONH ₂	N
P6	Adenovirus type 2/5 E1a; 282–289 (16)	C G G <u>L S S K R P R P</u> COOH	N
P7	Adenovirus type 2/5 E1a; 282–289 (16)	C G Y G <u>L S S K R P R P</u> G CONH ₂	C > N
P8	Polyomavirus large T; 279–286 (22)	C G <u>P P K K A R E D</u> CONH ₂	C > N
P9	SV40 large T (K→T); 126–132 (10)	C G G <u>P K T K R K V</u> G CONH ₂	C ≫ N
P10	Adenovirus type 7 E1a; 254–261 (11)	C G G <u>S T R K L P R Q</u> COOH	C ≫ N
P11	Adenovirus type 7 E1a; 254–261 (11)	C G Y G <u>S T R K L P R Q</u> G CONH ₂	C
P12	SV40 VP1; 1–11 (26)	<u>A P T K R K G S S P G</u> C CONH ₂	C
P13	SV40 VP1; 1–8 (26)	C G Y G <u>A P T K R K G S S</u> CONH ₂	C
P14	<i>Drosophila</i> Antennapedia; 26–33 (17)	C G Y G <u>L T R R R R I E</u> G CONH ₂	C
P15	Human p53; 369–377 (9)	C <u>L K S K K G O S T</u> CONH ₂	C
P16	Human p53; 316–324 (9)	C G G <u>P Q P K K K P L D</u> CONH ₂	C
P17	Human immunodeficiency virus gp120; 504–511 (21)	C G G <u>A P T K A K R R</u> CONH ₂	C
P18	<i>S. cerevisiae</i> MATα2-encoded protein; 1–13 (8)	<u>M N K I P I K D L L N P Q</u> C CONH ₂	C

^a The underlined sequences were derived from the indicated proteins. The coordinates are based on the reference(s) listed for each peptide.

^b N and C refer to nuclear and cytoplasmic localizations, respectively. Our assessment of localization was qualitative and was derived from Fig. 1 and 2 and many other figures (not shown) for the same peptide conjugates. N, More than 90% of the CSA protein in the nucleus; C > N, less than 50% in the nucleus; C ≫ N, less than 10% in the nucleus; C, no detectable CSA in the nucleus.

analysis. All of the conjugates used in this study had a substitution level of 4.5 to 6 peptides per carrier molecule.

Microinjection and immunofluorescence. HeLa cells were grown in McCoy medium (GIBCO Laboratories, Grand Island, N.Y.) on no. 2 cover slips which had been premarked with a diamond pencil to identify and localize injection sites. Cells were microinjected in the growth medium at 22°C by the method of Graessmann and Graessmann (7) with a Leitz micromanipulator and an inverted phase-contrast microscope. Glass needles were made on a vertical pipette puller (David Kopf Instruments, Tujunga, Calif.). After injection, the cells were returned to a CO₂ incubator for the times indicated. Cover slips were then washed three times with ice-cold phosphate-buffered saline and fixed with –20°C methanol-acetone (1:3) for 10 min and dried. CSA was detected by indirect immunofluorescence with rabbit immunoglobulin G against CSA (Organon Teknika) and subsequently with affinity-purified, rhodamine-conjugated goat anti-rabbit immunoglobulin G (Pel-Freez Biologicals, Rogers, Ark.). The injected cells were viewed under a Zeiss epifluorescence microscope.

RESULTS

Several known targeting sequences mimicked by synthetic peptides. The abilities of 18 synthetic peptides to target CSA to the nucleus were assessed following microinjection of the peptide-protein conjugates into the cytoplasm of HeLa cells. Each peptide was coupled to CSA with a bifunctional cross-linking reagent. The reagent, *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester, was first reacted with free amino groups on the protein. This complex was then isolated and subsequently allowed to react with a cysteine sulfhydryl group on the peptide. Peptides were synthesized with the cysteine residue at one end or the other to simulate the N or C terminus of the protein we were attempting to mimic. For sequences derived from the internal region of a protein, we typically used resins for peptide synthesis which produced an amide (CONH₂) in place of a carboxyl group (COOH) at the C terminus of the peptide. Glycine residues were also

occasionally used as spacers to help mimic internal sequences.

Several of the nuclear protein sequences chosen for synthesis (P1, P2, P3, P6, P7, P8, P12, and P13; Table 1) were based on the known nuclear targeting regions of those proteins as determined by recombination methods. One of the peptides, P1, is nearly identical to a peptide reported by Lanford et al. (14) to facilitate nuclear transport following conjugation to a variety of proteins. The P1 peptide, based on an SV40 T-antigen sequence (10), was highly effective at targeting CSA to the nucleus. A single-residue change of a lysine to a threonine largely eliminated targeting ability (Table 1, P9 versus P1). This is consistent with results obtained in experiments with peptide conjugates (6, 14) and recombinants (10).

Two regions of the polyomavirus T antigen have been shown to be effective at nuclear targeting, although to differing extents, on the basis of experiments with recombinants. One of these sites, consisting of residues 189 to 196, was able to efficiently target a polyomavirus T-antigen-pyruvate kinase fusion protein to the nucleus (22). This sequence was mimicked in our experiments by peptide P2, which was also found to be very effective at nuclear targeting (Table 1 and Fig. 1). A second region of polyomavirus T antigen, residues 279 to 286, was ineffective at targeting a fusion protein to the nucleus but was found to have some targeting ability on the basis of deletion analysis (22). A peptide, P8, based on this second site was able to target CSA to the nucleus but much less effectively than peptide P2 (Table 1 and Fig. 1). The two polyomavirus peptide sequences differ significantly. The less effective peptide has three instead of four positively charged residues and, in addition, has two negatively charged residues immediately adjacent to the positively charged region.

The nine C-terminal amino acids of protein E1a of adenovirus type 2/5 have been shown to be necessary for rapid nuclear localization of that protein (16). If these residues are deleted, the protein still enters the nucleus, presumably owing to its relatively small size, but at a much reduced rate.

FIG. 1. Distribution of peptide-CSA conjugates 45 min after microinjection into the cytoplasm of HeLa cells. Peptide conjugates were visualized by indirect immunofluorescence staining as described in Materials and Methods. The number in each panel refers to a peptide designation in Table 1.

In addition, fusion of the five C-terminal residues of E1a to the C terminus of galactokinase causes the resulting recombinant protein to be localized in the nucleus (16). A synthetic peptide (P6) containing the eight C-terminal adenovirus type 2/5 protein E1a residues was very effective at targeting CSA to the nucleus (Table 1). This same sequence, when synthesized with a flanking C-terminal glycine (P7), lost much of its targeting ability (Table 1 and Fig. 1). Neither of the two E1a peptides was identical to the native protein, however, since we substituted a serine for a cysteine in the original sequence (LSSKRPRP instead of LSCKRPRP). This was necessary,

since both peptides are conjugated through an N-terminal cysteine sulfhydryl. This substitution, however, was outside of the five-amino-acid region necessary for nuclear localization of the fusion protein.

The 11 N-terminal amino acids of SV40 protein VP1 have been found to be sufficient to direct a fusion protein to the nucleus (26). Two peptides were synthesized with the VP1 sequence. In the first peptide (P12), the 11-amino-acid VP1 sequence was positioned at the N terminus to mimic the native protein. It was necessary, however, to replace cysteine 9 in the original sequence with a serine. This was done to

ensure that conjugation would take place only through a cysteine residue added at the C terminus of the peptide, as with the adenovirus type 2/5 E1a peptides. For the second peptide (P13), we used only the first eight amino acids, thereby avoiding the cysteine-serine switch. In addition, the sequence was positioned at the C terminus of the peptide, since all of the peptides capable of directing nuclear transport were oriented in that way. Neither of the two VP1 peptides was able to target CSA to the nucleus (Table 1 and Fig. 1).

Peptides chosen for similarity to known targeting sequences.

Possible nuclear targeting sequences were chosen from known nuclear proteins in which the localization signals had not been precisely defined. Our choices were based on the presence of clustered basic residues, which is a common theme in the previously identified targeting peptides. Three peptides (P3 to P5), derived from sequences in nucleoplasmin (4) and lamin L₁ (12) from *X. laevis*, and the human *c-myc* protein (24) were very effective at nuclear targeting (Table 1 and Fig. 1). The nucleoplasmin signal was slightly less effective than the others, however, since we always observed a small amount of cytoplasmic staining with this conjugate (Fig. 1). Fusion protein analysis localized the nucleoplasmin nuclear targeting signal to three possible overlapping regions. These are between amino acids 161 and 172 (1), amino acids 156 and 172 (4), and amino acids 153 and 169 (4). Our peptide, P3, covers positions 164 to 172. The nuclear targeting signal of lamin L₁ has not been previously investigated, but a peptide almost identical to our *c-myc* peptide has been described by Dang and Lee (2). They also found that this sequence successfully targets a carrier protein to the nucleus.

The C-terminal region of nuclear protein E1a from adenovirus type 7 (11) was synthesized on the basis of the success of the same region of E1a from adenovirus type 2/5. The two E1a sequences differ slightly, and only the adenovirus type 2/5 sequence has been previously investigated for nuclear targeting ability (16). A peptide based on the eight C-terminal amino acids of E1a from adenovirus type 7 (P10) was only marginally able to target CSA to the nucleus following microinjection into the cytoplasm (Table 1 and Fig. 1). The same sequence, synthesized with a flanking C-terminal glycine (P11), had no nuclear targeting ability.

Four additional sequences were tested and found to be negative for nuclear transport. Sequences from the homeobox domain of the *Drosophila* Antennapedia gene product (17) and from human p53 (9), both of which are nuclear proteins (23, 25), were ineffective. Both proteins have other clusters of basic residues, however, and an alternate choice of sequence or a combination thereof may be required. Also, p53 is localized to the cytoplasm in nontransformed cells, suggesting that its localization to the nucleus depends on other factors (for a review, see reference 23). A peptide, P17, was also synthesized to mimic a region on the gp120 protein from the human immunodeficiency virus (21). This region contained a clustering of basic residues and shared the sequence APTK with the SV40 VP1 protein-targeting signal. CSA conjugated to this peptide was excluded from the nucleus. However, as described above, the VP1 sequence proved ineffective as well.

Yeast nuclear targeting signal. The thirteen N-terminal amino acids of the *Saccharomyces cerevisiae* MAT α 2-encoded protein are thought to be responsible for targeting that protein to the yeast nucleus (8). We synthesized a peptide with those 13 amino acids at the N terminus followed by a C-terminal cysteine for conjugation (P18). This peptide,

conjugated to CSA, was ineffective at targeting that protein to the nuclei of HeLa cells (Table 1 and Fig. 2).

Evidence of the targeting ability of a peptide by 45 min after injection. Conjugates of CSA and the peptides P1 to P6 entered the nucleus very rapidly. Most of each of these conjugates was detected in the nucleus at 15 to 30 min postinjection (data not shown). Only a small increase was observed between 30 and 45 min. Therefore, the 45-min time point was selected for all observations. To determine whether more time was needed for two of the peptides which showed either incomplete accumulation or absence of accumulation by 45 min, we extended the postinjection time up to 4 h. The nucleus did not permit entry, even after 4 h, to a protein without the appropriate nuclear targeting signal (i.e., P18; Fig. 2). A peptide (P9) with limited nuclear accumulation by 45 min did not show a significant increase in accumulation by 4 h (Fig. 2).

DISCUSSION

We found a high degree of correlation between nuclear transport of CSA facilitated by synthetic peptides and similar experiments using recombinant DNA to define the nuclear targeting region of a protein. In addition, several sequences which are similar to known targeting signals were also found to be effective at targeting a carrier protein to the nucleus. Peptides representing small regions of the SV40 and polyomavirus T antigens, adenovirus protein E1a, the human *c-myc*-encoded protein, and *X. laevis* nucleoplasmin and lamin L₁ were all effective at directing nuclear transport when covalently attached to CSA. Comparison of the sequences which resulted in nuclear transport with those that did not revealed a possible consensus sequence for peptide-directed nuclear transport. All of the peptides which induced full or partial nuclear localization (P1 to P8) fit the four-residue consensus sequence Lys-Arg/Lys-X-Arg/Lys. All of the peptides which do not direct nuclear transport (P9 to P18), including the SV40 VP1 sequence, do not fit this sequence. The X position is filled in our peptides by lysine, arginine, proline, valine, or alanine. Not all amino acids can substitute, however, since there is a peptide described by Lanford et al. (15) with an asparagine in the X position which does not direct a carrier protein to the nucleus.

The fact that the VP1 sequence did not direct transport and did not fit our consensus sequence suggests the possibility that there is more than one class of nuclear targeting signal, only one of which can be mimicked by a peptide conjugate. However, in VP1 (P12), we replaced cysteine 9 with a serine so that the cysteine placed in position 12 would be the only one used in the conjugation of the peptide to the carrier protein. It may be that this signal requires the cysteine at position 9, perhaps to induce a specific conformation necessary for its function.

An *X. laevis* nucleoplasmin-derived peptide, P3, which was found to be a functional transport signal in our experiments, is shorter than the signal defined for this protein by gene fusion (1, 4). The sequence we chose to mimic includes our proposed consensus sequence and two amino acids on either side. Additional residues, including several basic amino acids on the N-terminal side, were necessary to direct a pyruvate kinase-nucleoplasmin fusion protein to the nucleus (4). However, all constructs which included the sequence we used but did not target the fusion protein to the nucleus also included three acidic amino acids on the C-terminal end of the sequence. The precise sequence we chose was not used in the fusion experiments.

FIG. 2. Time dependence of cellular distribution of peptide conjugates. Two peptides with little (P9) or no (P18) ability to target CSA to the nucleus by 45 min after injection were tested for the time dependence of their distribution in the cell. Conjugates were microinjected into the cytoplasm of HeLa cells, which were then incubated at 37°C for the times indicated before fixation and immunofluorescence staining.

A peptide representing the yeast *MAT α 2* nuclear localization signal did not target CSA to the nuclei of mammalian cells in our study. The 13 N-terminal residues of this protein were sufficient to target a fusion protein to the nuclei of yeast cells (8). Our peptide was constructed with the same 13 amino acids positioned at the N terminus of the peptide. It may be that the signal requirements in *S. cerevisiae* and mammalian cells are not completely interchangeable.

With synthetic peptides, it has been possible to define the minimum requirements for nuclear targeting. Both synthetic peptide and gene fusion analyses, however, showed that the sequences surrounding this minimum region can have a major influence on targeting ability. For example, the C-terminal adenovirus type 2/5 E1a sequence works well when presented at the C terminus of the peptide but works poorly when flanked by a glycine residue. Similarly, the nucleoplasmin signal works well as a short synthetic peptide but gives variable results when various portions of the flanking sequence are included in fusion protein experiments (1, 4). Analysis with synthetic peptides allows for rapid dissection of sequences such as these. When necessary, small and precise changes can be made with few constraints. Most importantly, however, it is apparent that a wide variety of putative targeting signals can be evaluated without access to or manipulation of DNA clones.

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