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Effect of Basic and Nonbasic Amino Acid Substitutions on Transport Induced by Simian Virus 40 T-Antigen Synthetic Peptide Nuclear Transport Signals

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A previous study demonstrated the ability of a synthetic peptide homologous to the simian virus 40 T-antigen nuclear transport signal to induce the nuclear transport of carrier proteins and the dependence of peptide-induced transport on a positive charge at the lysine corresponding to amino acid 128 of T antigen. In this investigation synthetic peptides were utilized to examine the effect on transport of amino acid substitutions within the T-antigen nuclear transport signal. Nuclear transport was evaluated by immunofluorescence after microinjection of protein-peptide conjugates into the cytoplasm of mammalian cells. Substitution of other basic amino acids at position 128 revealed a hierarchy for nuclear transport. The rate of nuclear transport was most rapid when a lysine was at position 128 followed in descending order by arginine, D-lysine, ornithine, and *p*-aminophenylalanine. Peptide-induced nuclear transport was dependent upon a positively charged amino acid at positions 128 and 129, since substitutions of neutral asparagines at these positions abolished transport. However, partial transport was observed with the peptide having an asparagine at position 128 when a high number of peptides were conjugated to the carrier protein.

Eucaryotic cells have evolved a system of membranous organelles for compartmentalizing specialized functions within the cell. One requirement for a complex system of organization is a mechanism for the accumulation of proteins at their site of function. The involvement of signal sequences in protein transport to various organelles is well documented (for reviews see references 8, 20, and 25). The movement of proteins from the cytoplasm to the nucleus is accomplished in part, if not entirely, by translocation across the nuclear pore complex (7). The nuclear pore represents a complex proteinaceous structure spanning the double nuclear membrane and provides an aqueous milieu in which protein transport across the nuclear membrane can occur. The nuclear pore channel appears to be open to diffusion of small molecules, whereas a mechanism of selective entry governs translocation of large proteins (for reviews see references 4, 5, and 20). The capacity for selective entry is retained by the mature protein, since extracted nuclear proteins can accumulate in the nucleus upon reintroduction into the cytoplasm of recipient cells. The diffusion of proteins across the nuclear membrane decreases with increasing size; the diffusion of ovalbumin (43,000 molecular weight) is retarded and bovine serum albumin (68,000 molecular weight) is excluded (17, 21, 22). Whether most smaller proteins traverse the nuclear pore by diffusion or specific transport cannot be ascertained until the mechanisms of specific transport are better understood. No apparent requirement exists for the retention of large nuclear proteins, since diffusion out of the nucleus is restricted as well (17).

During the past few years, the amino acid sequences responsible for the selective entry of many nuclear proteins have been determined. The nuclear transport signal of simian virus 40 (SV40) T antigen represents the prototypic signal sequence and is comprised of a highly basic stretch of amino acids (Pro-Lys-Lys-Lys-Arg-Lys-Val) (12, 16). An absolute requirement exists for a positively charged amino acid at the position of the second lysine, with the surrounding basic residues having a lesser effect on transport (3, 12, 16). Fusion proteins containing this sequence are efficiently transported to the nucleus (13). Furthermore, synthetic peptides homologous to the SV40 T-antigen nuclear transport signal can induce the transport of a variety of carrier proteins (9, 17). Proteins as large as ferritin (465,000 molecular weight) are efficiently transported by the synthetic peptides, and the rate of transport is influenced by both the size of the carrier protein and the number of signal peptides per molecule of carrier protein (17). Nuclear transport signals similar to that of the SV40 T antigen have been identified in several proteins including polyomavirus large-T antigen (23), SV40 VP1 (28), *Xenopus laevis* N1/N2 protein (14), and *X. laevis* nucleoplasmin protein (2, 6). However, other proteins have sequences involved in nuclear transport that have little or no obvious homology to the SV40 T-antigen signal, including yeast proteins MATα2 (10), GAL4 (26), and L3 (19) and the adenovirus E1a protein (18). Analysis of the polyomavirus T antigen revealed that proteins may have more than one domain involved in nuclear transport (23).

In this report we describe a systematic survey of the effect of amino acid substitutions on peptide-induced nuclear transport with synthetic peptides homologous to the SV40 T-antigen nuclear transport signal. Synthetic peptide-carrier protein conjugates were microinjected into the cytoplasm of mammalian cells, and nuclear transport was evaluated by immunofluorescence at various times after microinjection. A hierarchy of transport rates was observed when various basic amino acids were substituted for the lysine corresponding to amino acid 128 of T antigen. Substitution of a neutral asparagine at positions 128 and 129 demonstrated that peptide-induced nuclear transport was dependent upon a positively charged amino acid at both of these positions.

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MATERIALS AND METHODS

Peptide synthesis. Transport signal peptides were synthesized on a glycyl-Merrifield resin by using a Biosearch Sam Two automated peptide synthesizer as described previously (17). Peptides were purified by reversed-phase high-pressure liquid chromatography and checked by amino acid analysis. Ornithine and *p*-aminophenylalanine were identified and quantitated in unknown samples after calibration of the amino acid analyzer with the appropriate standards. Purity was assessed by the presence of a single peak by analytical reversed-phase high-pressure liquid chromatography, the correct amino acid composition, and the presence of the predicted parent ion by fast-atom bombardment-mass spectroscopy.

Preparation of protein-peptide conjugates. Synthetic peptides were conjugated to carrier proteins with the heterobifunctional cross-linking agent *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, Ill.) by a modification of a previously described procedure (17). Carrier proteins were derivatized with *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester in preparative batches and divided into multiple equal samples for reaction with the various peptides to increase uniformity of the protein-peptide conjugates. Fatty acid-free bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, Mo.), and mouse immunoglobulin G (IgG) was prepared from the hybridoma cell line secreting the monoclonal antibody PAb 405 (11). Previously unconjugated peptide was removed only by dialysis in phosphate-buffered saline overnight at 4°C; however, during the performance of this study an additional step was included to remove unconjugated peptide that adhered to the carrier proteins. This step involved repeated concentration and dilution of conjugates with Centricon-30 microconcentrators (Amicon Corp., Danvers, Mass.).

Estimation of protein-peptide coupling ratios. Amino acid analyses of protein-peptide conjugates were used to obtain a number-average ratio of signal peptides to carrier (1). The amino acid compositions of BSA and IgG were obtained for three or more samples each, and the final composition was determined by averaging the results. Peptide-carrier conjugates were then analyzed and compared with the carrier results. The increase in the ratios of residues contained in the peptides to those which are absent was used to calculate the ratio of peptide to carrier protein in each conjugate sample (see Results and Table 2). Only amino acids that were stable under hydrolysis conditions were used in the analyses.

Microinjection. TC-7 cells, a stable line of African green monkey kidney cells (24), were grown on glass cover slips etched with a diamond pen to facilitate localization of injected cells. Microinjections were performed as previously described (17). Approximately 100 cells were injected per experimental variable, and all experiments were conducted at least two times. Microinjected cultures were incubated at 37°C for various times before harvest. Immunofluorescence was performed as previously described (17) with antibodies directed toward the carrier proteins.

RESULTS

Selection of amino acid substitutions for T-antigen nuclear transport signals and potential effects on conformation. The use of synthetic peptides for the analysis of nuclear transport presents a diversity of approaches not possible with recombinant proteins. In particular, the types of amino acid substitutions available with peptide chemistry can help better define the requirements for interaction between a ligand and a receptor. The ligand in this investigation is a 13-mer synthetic peptide containing seven amino acids homologous to SV40 T antigen. The receptor for nuclear transport signals is an undefined component of the cellular machinery involved in the transport of proteins from the cytoplasm to the nucleus. A functional assay for ligand-receptor interaction has been developed that involves cytoplasmic microinjection of carrier protein-signal peptide conjugates and evaluation of transport by immunofluorescence staining for the carrier protein (17). Previous studies have demonstrated the requirement for a positive charge at the position of the second lysine (amino acid 128) in the T-antigen nuclear transport signal (Pro–Lys–Lys-128–Lys–Arg–Lys–Val) (9, 12, 16, 17). To evaluate the effect of conformational changes in the transport signal when a positive charge is maintained at position 128, we have substituted several positively charged nonprotein amino acids for Lys-128 in the wild-type (WT) signal peptide. The amino acid sequences of the peptides employed in this study are illustrated in Table 1. The WT peptide contains the seven-amino-acid core sequence for nuclear transport of T antigen with two glycine residues at the carboxyl terminus for spacer effect and the sequence Cys-Gly-Tyr-Gly at the amino terminus as a spacer with a tyrosine for iodination and a terminal cysteine for coupling to carrier proteins through the free sulfhydryl group (17). The positively charged amino acids substituted for Lys at position 128 include arginine (Arg), D-lysine (D-Lys), ornithine (Orn), and para-aminophenylalanine (PAF). The neutral asparagine (Asn) at 128 mimics the mutation present in SV40 (cT)-3 that encodes a T antigen that is defective in transport to the nucleus (12). In addition, a peptide with Asn at position 129 was synthesized to examine the importance of the other positively charged amino acids for peptide-induced transport.

TABLE 1. Modified T-antigen signal peptides

Peptide	Amino acid sequence
WT	Cys-Gly-Tyr-Gly-Pro-Lys-Lys[a]-Lys-Arg-Lys-Val-Gly-Gly
cT	-Asn-
Arg	-Arg-
D-Lys	-D-Lys-
Orn	-Orn-
PAF	-PAF-
Asn-129	-Asn-

[a] Amino acid no. 128 for SV40 T antigen. The amino acids listed below this Lys indicate substitutions at position 128, except for the Asn at position 129.

The potential effect of these substitutions on the conformation of the signal peptide can be envisioned by examining the structures of the amino acids (Fig. 1). Lys has a four-carbon aliphatic side chain with an epsilon amino group. The guanidinium group on Arg complicates the assessment of transport capacity to a single structure, since the positive charge is shared via resonance with all three nitrogen atoms. Orn is the closest structural analog to Lys with a side chain that is one methylene group shorter than Lys. The bulky aryl amine present on PAF should result in substantial changes in the conformation of the peptide. D-Lys is the optical isomer of L-Lys; although it has the same side chain as L-Lys, the positively charged amino group will be in a different spatial orientation with respect to the side chains of the adjacent positively charged amino acids. Asn lacks a positively charged group and thus compares the importance of a positive charge with requirements

for the conformational presentation of positive charges. Although precise predictions cannot be made concerning the conformations of the various synthetic peptides, it is apparent that the positive charge at position 128 is presented in several different contexts.

Preparation and analysis of carrier protein-peptide conjugates. The accurate comparison of several peptides for their capacity to induce transport of carrier proteins to the nucleus imposed several criteria upon the evaluation of the carrier protein-peptide conjugates. Our previous study suggested that the number of peptide signals per molecule of carrier protein and the size of the carrier protein could influence the rate of transport to the nucleus (17). Thus, two different carrier proteins were selected to eliminate potential carrier effects, and several methods were used to evaluate the peptide/carrier protein ratio in conjugates. BSA (68,000 molecular weight) and a monoclonal IgG (150,000 molecular weight) were chosen as carrier proteins because they differ considerably in size and both could be transported to the nucleus with rapid kinetics by the WT peptide. The methods used for the estimation of peptide/carrier protein ratios included iodination of a small percentage of the peptide at the tyrosine residue and measurement of the radioactivity incorporated into the carrier protein, estimation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the

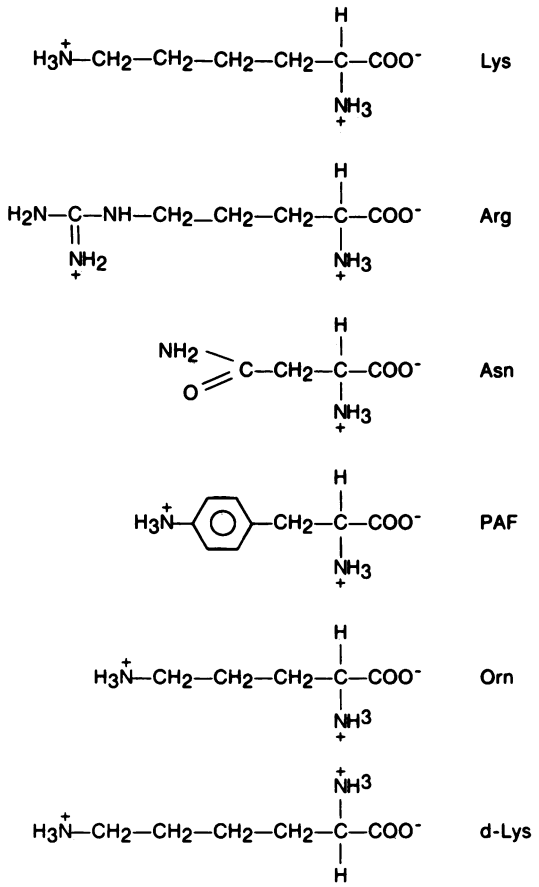

FIG. 1. Structures of amino acids substituted for Lys-128 of the T-antigen synthetic peptide nuclear transport signal. The structures of the amino acids employed in this investigation are presented to emphasize the different contexts for presentation of the positive charge at position 128 of the synthetic peptide nuclear transport signal.

TABLE 2. Determination of peptide/carrier protein ratio for the Orn-IgG conjugate by amino acid analysis

Amino acid[a]	IgG (X)[b]	IgG-peptide (Y)[c]	Peptide (A)[d]	Mol of peptide/mol of IgG[e]
Asp	115	5.705	0	
Glu	127	6.265	0	
Gly	79	5.096	4	6.3
Ala	66	3.008	0	
Val	95	4.890	1	5.2
Ile	42	2.093	0	
Leu	77	3.827	0	
Phe	47	2.377	0	
His	26	1.138	0	
Lys	78	4.444	3	4.3

[a] Residues such as Thr, Ser, Tyr, etc., which are partially destroyed during hydrolysis, were not used in the calculations.

[b] X indicates the experimental amino acid composition of the monoclonal IgG used for coupling. Values represent the average of at least three analyses. The total number of residues not found in the peptide are added ($\Sigma_1 = 500$).

[c] Y indicates the experimental composition found for the IgG-peptide conjugate. The total number of residues found in the carrier IgG only are added ($\Sigma_2 = 24.413$). A factor, Z, was calculated from this ratio (Σ_1/Σ_2) ($Z = 20.48$).

[d] A is the theoretical molar ratios of Gly, Val, and Lys per mole of the ornithine-containing peptide.

[e] The calculated molar ratio of peptide to IgG in the conjugate using the expression: $[(Z \cdot Y) - X]/A$ = moles of peptide per mole of IgG, where Z, Y, X, and A are as defined above (e.g., for Gly, $X = 79$, $Y = 5.096$, $Z = 20.48$, and $A = 4$). The average number of moles of peptide per mole of IgG obtained by this method was compared with that obtained by the amount of ornithine present in the sample (0.2481 nmol) multiplied by the factor Z (20.48). This value (5.1) agrees with that (5.3) obtained by the total amino acid analysis.

increase in apparent molecular weight induced by the addition of peptide to the carrier protein, and calculation by amino acid analysis of the increase in mass of specific residues found in the peptide in comparison to residues absent in the peptide. The results from incorporation of iodinated peptide into the carrier protein and amino acid analysis of the conjugates initially gave values much greater than those obtained by retardation of mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The data suggested that free peptide could not be completely removed from the conjugates by extensive dialysis, presumably due to electrostatic or other attractive forces which bind peptides to carrier proteins. Subsequent analyses demonstrated that repeated concentration and dilution with Centricon microconcentrators removed the residual free peptide that remained associated with the carrier proteins after dialysis. We find this to be a persistent problem with other conjugate preparations as well.

Comparison of the peptide/carrier protein ratios obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid analysis for conjugates prepared by dialysis and multiple cycles of Centricon concentration yielded very similar results. The values presented in Table 2 demonstrate the methodology utilized for determining coupling ratios by amino acid analysis, whereas Table 3 presents the coupling ratios determined for each of the conjugates employed in this investigation.

The data presented in this report were obtained by using one set of IgG-peptide conjugates and two sets of BSA-peptide conjugates, although multiple other conjugates were prepared and evaluated during the course of the investigation with similar results. Two different sets of BSA conjugates were evaluated because the Arg and Asn-129 peptides were synthesized and conjugated after the transport studies with the other peptides had been completed. However, each

TABLE 3. Transport times for peptide-carrier protein conjugates

Conjugate	Coupling ratio[a] (peptides/carrier)	Nuclear localization	Transport time[b]
IgG-WT	4.5	+	p15 min
IgG-cT	3.9	−	
IgG-D-Lys	5.7	+	3 h
IgG-Orn	5.3	+	>6 h
IgG-PAF	4.3	−	
BSA-WT	15.5	+	15 min
BSA-cT	13.6	±	
BSA-D-Lys	14.4	+	3 h
BSA-Orn	14.9	+	>6 h
BSA-PAF	11.7	−	
BSA-WT	3.1	+	15 min
BSA-Arg	4.2	+	1 h
BSA-cT	6.6	−	
BSA-Asn-129	4.8	−	

[a] Peptide-carrier protein conjugates were prepared as described in the text. Coupling ratios were determined by amino acid analysis.

[b] Peptide conjugates were microinjected into the cytoplasm of TC-7 cells, and cultures were returned to a 37°C incubator for various times before evaluation of transport by immunofluorescence.

set of conjugates was evaluated by comparison with WT and cT peptide conjugates prepared at the same time.

Effect of basic amino acid substitutions on peptide-induced transport. The capacity for various peptides to induce nuclear transport of carrier proteins was appraised by determining the time required for the carrier protein to become localized exclusively to the nucleus after microinjection into the cytoplasm. The IgG-WT peptide conjugate became localized exclusively to the nucleus within 15 min after microinjection into the cytoplasm of TC-7 cells (Fig. 2A). The IgG–D-Lys conjugate had the next most rapid rate of nuclear accumulation. After 1 h of incubation at 37°C, a significant percentage of the fluorescence was observed in the nucleus (Fig. 2B), and at 3 h the IgG–D-Lys conjugate was localized entirely within the nuclear compartment (Fig. 2C). The time required for complete nuclear transport of the IgG-Orn conjugate was greater than 6 h. At 1 h after microinjection, very little fluorescence was observed in the nucleus (Fig. 2D). Significant nuclear accumulation had occurred at the 3-h time point (Fig. 2E); although in some cells fluorescence was completely nuclear 6 h after microinjection, many cells displayed both nuclear and cytoplasmic fluorescence (Fig. 2F). To determine whether the IgG-Orn conjugate could become entirely nuclear after prolonged incubation, microinjected cultures were incubated overnight (16 h) at 37°C. Caution should be employed in the interpretation of results from experiments involving extended incubation times, since the possibility of degradation of the carrier protein-peptide conjugate cannot be explored due to the small volume microinjected into mammalian cells (50 to 100 fl). However, the IgG-Orn conjugate was exclusively nuclear in localization after 16 h of incubation (Fig. 2G). The IgG-PAF conjugate was the only conjugate containing a basic residue at position 128 that was not capable of significant nuclear transport. The fluorescence was restricted to the cytoplasm at the 1-h time point (Fig. 2H). After 6 h of incubation at 37°C, a small amount of fluorescence was associated with the nucleus, but the fluorescence was not evenly distributed throughout the nucleus as was seen with the other conjugates; instead, several small patches of fluorescence were observed (Fig. 2I). Possibly, the IgG-PAF conjugate became associated with the outer nuclear membrane rather than translocating across the nuclear membrane.

The rates of transport for BSA conjugates of WT, D-Lys, Orn, and PAF were very similar to those obtained for IgG (Table 3). In addition to these conjugates, a BSA-Arg conjugate was prepared. The BSA-Arg conjugate had a lower coupling ratio than the other BSA conjugates; however, it was compared with a BSA-WT conjugate that was prepared at the same time and had a similar coupling ratio (Table 3). There was no readily apparent difference in the transport times for the BSA-WT conjugates with coupling ratios of 3.1 and 15.5. Within 15 min after microinjection nuclear transport was complete for both conjugates; however, shorter time points were not examined. The BSA-Arg conjugate had a coupling ratio of 4.2. The BSA-Arg conjugate was only partially localized within the nucleus 15 min after microinjection, but at the 1 h time point transport to the nucleus was essentially complete (data not shown). Although the BSA-Arg conjugate had a lower coupling ratio than the other conjugates, only the WT conjugate had a greater rate of transport to the nucleus.

These results suggest that minor changes in the conformation of the signal peptide can have substantial effect on the rate of transport, and that a positively charged amino acid at position 128 is not sufficient to ensure transport to the nucleus.

Effect of nonbasic amino acid substitutions on peptide-induced transport. In a previous study (17) we examined BSA and IgG as protein carriers for transport with the cT peptide, which has a neutral Asn residue at position 128. Significant transport to the nucleus was not demonstrated with these conjugates; however, the conjugates had relatively low coupling ratios, and transport was not evaluated beyond a 2-h time point. In this investigation, we wished to extend those observations by examining BSA-cT conjugates with a greater coupling ratio than those previously employed and by evaluating transport after prolonged incubation. In addition, a peptide was synthesized with an Asn at position 129 to determine the effect of nonbasic amino acid substitutions on other positions in the transport signal.

The BSA-cT and BSA–Asn-129 conjugates were microinjected into the cytoplasm of TC-7 cells and examined by immunofluorescence after incubation of 2, 4, and 6 h at 37°C. The BSA-cT conjugate with a coupling ratio of 6.6 peptides per molecule of BSA did not exhibit a significant capacity for transport at any time point examined. Fluorescence was restricted entirely to the cytoplasm at 2 and 6 h (Fig. 3A) after microinjection into the cytoplasm. In contrast, the BSA-cT conjugate with a coupling ratio of 13.6 displayed a limited ability to enter the nucleus. At the 2-h time point a very weak fluorescence was apparent in the nucleus (Fig. 3B), and the intensity of the fluorescence increased to an easily detectable signal after a 6-h incubation at 37°C (Fig. 3C). These results suggest that the cT peptide can be recognized by the cellular machinery involved in the transport of proteins to the nucleus, but that high coupling ratios are required to achieve demonstrable levels of transport.

The BSA–Asn-129 conjugate had a coupling ratio of 4.8 (Table 3) which was lower than both BSA-cT conjugates but higher than the BSA-WT and BSA-Arg conjugates that displayed rapid rates of transport to the nucleus. No significant capacity for transport was observed for the BSA–Asn-129 conjugate for any time point examined. Fluorescence was restricted entirely to the cytoplasm after 2 h of incubation, and this pattern was not significantly different after extending the incubation time to 6 h (Fig. 3D). These results indicate that substitution of an Asn at position 129 has the same deleterious effects on nuclear transport as

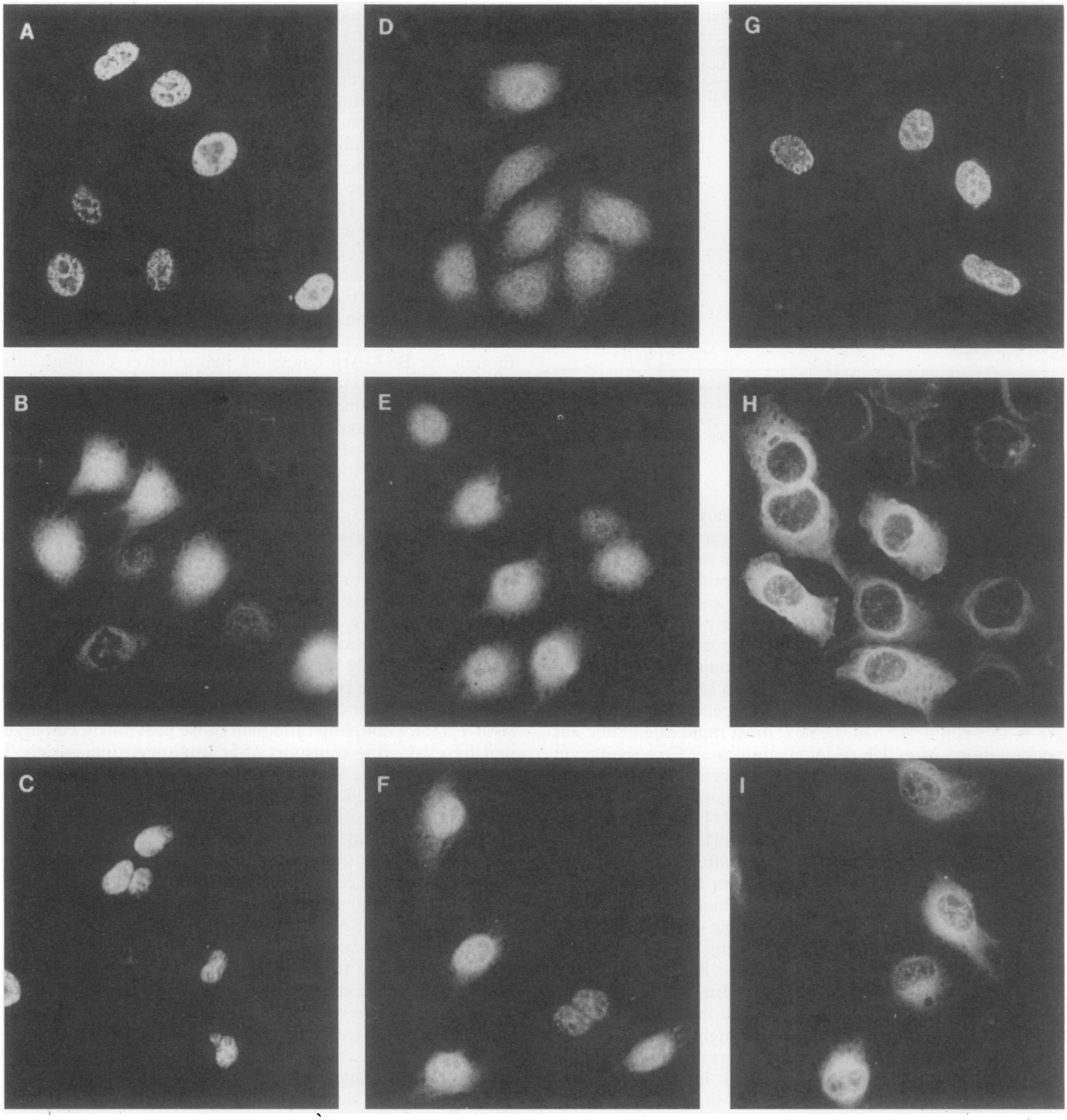

FIG. 2. Rate of transport for IgG-peptide conjugates. IgG conjugates with the various synthetic peptides were microinjected into the cytoplasm of TC-7 cells, and cultures were incubated at 37°C for various times before harvesting for immunofluorescence. Cells microinjected with the IgG-WT conjugate were harvested at 15 min (A). Cells microinjected with the IgG–D-Lys conjugate were harvested at 1 h (B) and 3 h (C). Cells microinjected with the IgG-Orn conjugate were harvested at 1 h (D), 3 h (E), 6 h (F), and 16 h (G). Cells microinjected with the IgG-PAF conjugate were harvested at 1 h (H) and 6 h (I).

substitution of an Asn at position 128, suggesting that a strong requirement exists for positively charged amino acids at both positions 128 and 129.

DISCUSSION

The initial reports that synthetic peptides homologous to the SV40 T-antigen nuclear transport signal could induce the transport of carrier proteins (9, 17) suggested that synthetic peptides may have numerous potential uses in elucidating the mechanism of nuclear transport. In this investigation, we have utilized synthetic peptides in a novel approach to mutagenesis studies for the analysis of a receptor-ligand interaction. The synthetic peptide nuclear transport signals represent the ligands, and the receptor is an undefined component of the cellular machinery involved in the trans-

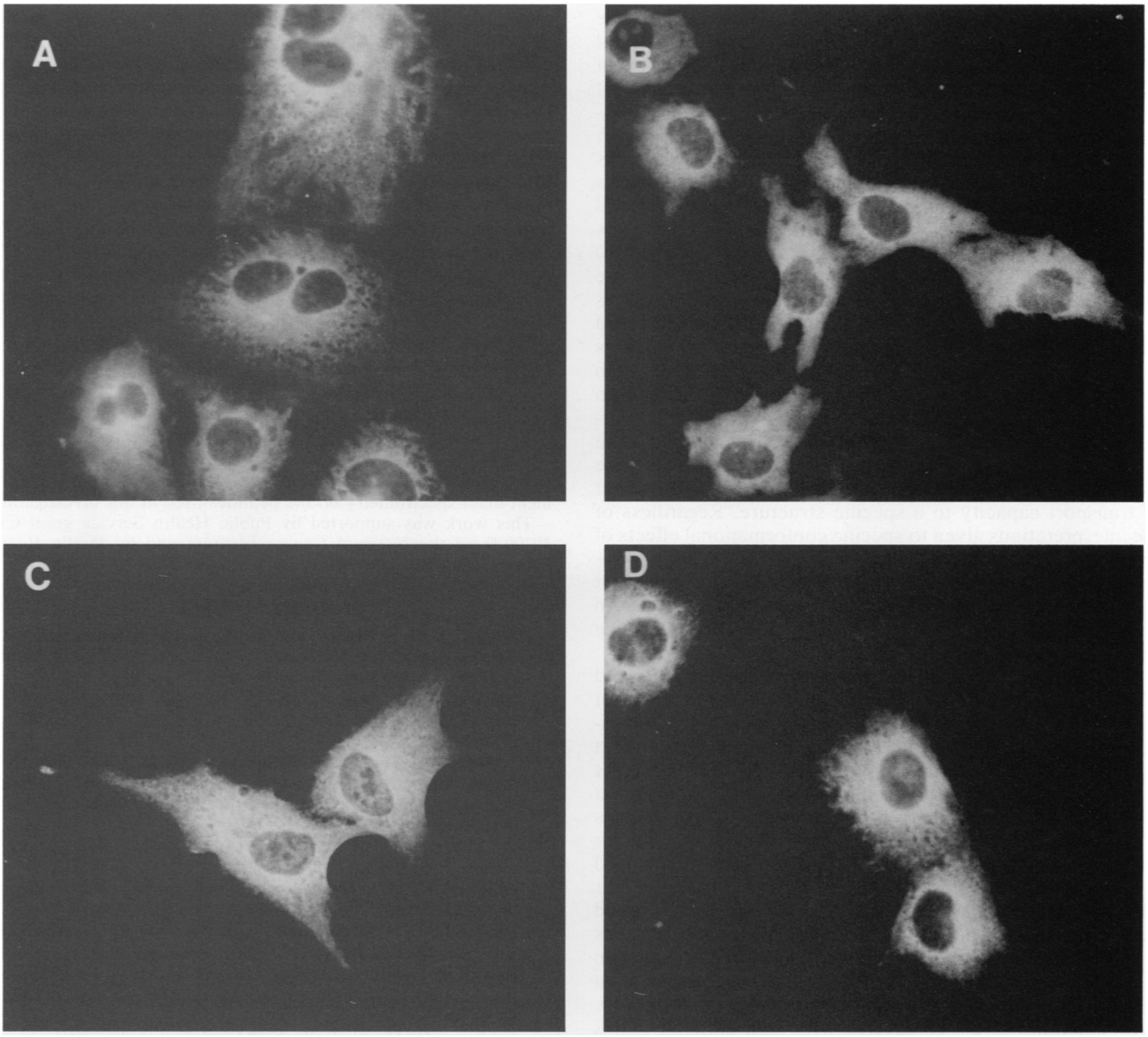

FIG. 3. Lack of efficient peptide-induced transport with Asn-substituted peptides. BSA-peptide conjugates were microinjected into the cytoplasm of TC-7 cells, and cultures were harvested for immunofluorescence after incubation for various times at 37°C. Cells microinjected with a BSA-cT (Asn-128) conjugate with a low coupling ratio (6.6 peptides per molecule of BSA) were harvested at 6 h (A). Cells microinjected with a BSA-cT conjugate with a high coupling ratio (13.6 peptides per molecule of BSA) were harvested at 2 h (B) and 6 h (C). Cells microinjected with a BSA–Asn-129 conjugate (4.8 peptides per molecule of BSA) were harvested at 6 h (D). BSA-cT and BSA–Asn-129 conjugates with a low coupling ratio had no significant capacity for transport, whereas the BSA-cT conjugate with a high coupling ratio demonstrated a low level of transport at the 6-h time point.

port of proteins from the cytoplasm to the nucleus. Previous studies on transport-defective mutants of T antigen suggested that a positively charged amino acid at position 128 of T antigen was essential for transport (3, 12, 16). These studies were limited in scope, because only two protein amino acids (Lys and Arg) have a positive charge under physiological conditions. In contrast, numerous positively charged nonprotein amino acids can be incorporated into synthetic peptides by the use of peptide chemistry. To better define the requirements for peptide-induced transport, the Lys of the WT peptide that corresponds to position 128 of T antigen was substituted with several different basic residues, including Arg, D-Lys, Orn, and PAF. Each peptide was evaluated for its ability to induce nuclear transport of BSA and IgG after microinjection of protein-peptide conjugates into the cytoplasm of mammalian cells. Each peptide had a characteristic rate of transport; the capacity for transport, in descending order, was L-Lys, Arg, D-Lys, Orn, and PAF.

Although the exact structure of the modified peptides has not been deduced, examination of the structures of the individual amino acids provides some insight into the requirements for transport. Since each of the peptides maintains a positive charge at position 128, the differences in the rate of transport should reflect an alteration in the conformation of the peptide and the spatial orientation of the positive charge with respect to the adjacent basic residues. The inability of PAF to induce the transport of carrier proteins is consistent with the hypothesis that a specific

conformation of the peptide is required for recognition. The bulky aryl amine group of PAF should induce substantial changes in the conformation of the peptide. The lack of transport for PAF demonstrates that a positive charge at position 128 is not sufficient to ensure transport. The relationship between amino acid substitutions and transport rates for the other peptides is less readily apparent. Orn has an aliphatic side chain one methylene group shorter than Lys and was initially considered to be the closest structural analog to L-Lys. Yet the transport rate for Orn conjugates (16 h) was slower than either Arg (1 h) or D-Lys (3 h) conjugates. This would suggest that the length of the side chain presenting the positive charge at position 128 is important for efficient receptor interaction. The increased rate of transport for D-Lys in comparison to Orn suggests that the length of the side chain may be more important than the spatial orientation of the positive charge at 128 in relation to the adjacent positive charges. The assessment of conformation effects of the Arg substitution is more complex. The guanidinium group shares the positive charge with all three nitrogen atoms via resonance and thus prevents assignment of transport capacity to a specific structure. Regardless of the interpretations given to specific conformational effects of amino acid substitutions, the preference for certain positively charged amino acids at position 128 for transport indicates that the receptor-ligand interaction involved in transport is restricted by both conformation and sequence dependent elements.

Substitution of neutral Asn residues at positions 128 and 129 revealed that the loss of a positive charge at either of these positions reduced transport to negligible levels. Previous mutagenesis studies with native T antigen suggested that position 128 was the most critical of the five adjacent positive charges. A T-antigen mutant with threonine at position 129 was capable of partial transport to the nucleus (12). Either peptide-induced transport is more demanding for a positive charge at position 129 or the Asn substitution used in this study is more detrimental to transport than the Thr substitution previously described. Additional studies would be required to determine the effects of nonbasic substitutions at each of the positively charged amino acids in the T-antigen signal peptide and whether certain nonbasic amino acid substitutions are more deleterious to transport than others.

Examination of cT conjugates with high coupling ratios demonstrated that the cellular proteins involved in nuclear transport are capable of recognizing the cT peptide. Although the level of transport observed with the high-coupling-ratio cT conjugates was low, the level of nuclear accumulation increased from 2 to 6 h postinjection. In addition, the level of nuclear accumulation for cT conjugates was demonstrably greater than that for PAF conjugates with similar coupling ratios. However, the significance of the low levels of transport observed for the cT conjugate with 13.6 peptides per molecule of BSA for biological studies on T-antigen mutants having a single defective transport signal is questionable.

The ability of short amino acid sequences to direct nuclear localization of nonnuclear proteins implies that these sequences are recognized by cellular protein(s) involved in transport in a fashion similar to receptor-ligand interactions. As more nuclear transport sequences are identified, the lack of a strict homology becomes increasingly apparent. Although nuclear transport signals similar to that of SV40 T antigen have been identified in some proteins (2, 6, 15, 23, 28), other proteins have signals with little or no obvious homology to that of T antigen (10, 18, 19, 26). One of the major questions to be resolved is whether a single cellular protein is capable of recognizing the dissimilar signal sequences or whether proteins containing different classes of signals initially enter the nuclear transport pathway by interaction with different cellular receptor proteins. The dramatic effect on transport observed when Orn is substituted for Lys in the T-antigen signal peptide might suggest that the signals highly divergent from T antigen are recognized by a different cellular protein. However, the lack of homology among signal sequences interacting with the SRP complex in transport across the endoplasmic reticulum (27) demonstrates the remarkable flexibility in cellular recognition processes. The use of synthetic peptides homologous to nuclear transport signals with divergent sequences may help to identify cellular proteins capable of interacting with these sequences and may elucidate some of the parameters involved in nuclear transport.

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