All Four Homochiral Enantiomers of a Nuclear Localization Sequence Derived from c-Myc Serve as Functional Import Signals*

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The information that targets a protein to the nucleus often consists of a short cluster of basic amino acids called a nuclear localization sequence (NLS). Since a wide range of sequences rich in basic amino acid residues function as NLSs, we postulated that an NLS-like sequence composed exclusively of d-amino acids might have biological activity. We synthesized peptides corresponding to the c-Myc NLS composed of either all l or d-amino acids, both in the forward and reverse order. We tested these peptides for nuclear import activity in a digitonin-permeabilized cell assay. All four peptide-bovine serum albumin conjugates localized to the nucleus with similar efficiency, and each conjugate competed for import with an SV40 large T antigen-derived NLS conjugate. Cross-linking experiments with free NLS peptides in HeLa cytosol indicated that each peptide bound to a protein that migrated at the molecular weight of importin a. Recombinant importin a, importin b, Ran, and NTF2 alone were sufficient to support the import of both l-form and d-form conjugates in permeabilized cells. This indicates that both d- and l-form NLS peptides use the same import machinery. Although the free d-forms of the NLS were proteolytically resistant in cytosol, the l-forms were rapidly degraded. To our knowledge, this is the first example of an intracellular pathway in which the receptor is insensitive to the chirality of the ligand.

A great deal of research is currently focused on the development of small synthetic peptides that mimic the biological activity of natural proteins. Small peptides offer a variety of advantages as therapeutic agents. Their small size facilitates absorption compared with large proteins (1), and they are more easily synthesized and, thus, less expensive to develop as therapeutic agents. Examples of successful small peptide drugs include anti-microbial agents (2), competitive inhibitors of angiotensin converting enzyme (3) and of the EPO receptor (4), and immunogenic agents (5, 6). With the number of published three-dimensional protein structures increasing, rational drug design based on molecular recognition may find its most direct application in the development of peptide mimetics.

One of the chief obstacles to the use of therapeutic peptide mimetics is their susceptibility to proteolysis (7). To address this problem, some research has been directed toward the development of d-amino acid peptide mimetics, which are resistant to proteolytic degradation (8). This resistance stems from the highly site-specific recognition required for protease binding (9). For example, a synthetic human immunodeficiency virus protease composed exclusively of d-amino acids can cleave d-peptide substrates but not l-peptide substrates (10). Similarly, the l-amino acid version of the protease cleaves l-peptides but not d-peptides. Unfortunately, the promise of this approach is compromised by the fact that relatively few d-amino acid peptides possess biological activity. Ironically, it is likely that the vice of d-peptides arises from their virtue; although proteases cannot recognize these peptides, neither can intended target proteins. A promising solution to the d-peptide conundrum was proposed by Chorev et al. (8), who suggested that d-reverse peptides might have greater biological activity than d-peptides by more closely mimicking the side chain to main chain orientation of l-peptides (for example, see Fig. 1B under "Results"). However, the efficacy of particular peptides synthesized as d or d-reverses is difficult to predict and must be determined empirically.

The nuclear protein import pathway offers an appealing model system for analyzing the relative activities of chiral peptides. Nuclear proteins are imported into the nucleus through aqueous channels that span the nuclear envelope called nuclear pore complexes. Although ions and molecules less than 20–40 Da can diffuse passively through the nuclear pore complexes, larger proteins are transported by saturable pathways that are energy- and signal-dependent. The signals that specify nuclear protein import (NLSs) are commonly short stretches of amino acids rich in basic amino acid residues, although other classes of NLSs have been described recently (11, 12). The initial step in the import of proteins containing basic amino acid-type NLSs occurs in the cytosol, where the NLS-containing proteins are bound to a receptor (variously called the NLS receptor, importin a, and karyopherin a (13). The substrate-receptor complex then associates with the cytoplasmic face of the nuclear pore complexes, and with the participation of other cytosolic factors, is transported through a gated channel in the nuclear pore complexes to the nuclear interior. The in vivo events of NLS-mediated nuclear import can be duplicated in an in vitro system using digitonin-permeabilized cells supplemented with cytosolic extracts and ATP (14). Transport in this in vitro assay is blocked by the same inhibitors that block in vivo import, is rapid, and is easily quantified.

Several properties of basic amino acid-type NLSs make them particularly suitable for chiral analysis. The minimal length required for activity is conveniently small (6 to 10 amino acids), and they retain their nuclear targeting function when synthe-

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1 The abbreviations used are: NLS, nuclear localization sequence; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; GTP·S, guanosine 5′-3′-O-(thio)triphosphate.
sized as discrete peptides and chemically coupled to carrier proteins such as BSA. The target receptors are apparently forgiving, for other than a preponderance of basic amino acids, neither the precise number nor sequence of amino acids defines a consensus required for function. For example, in the case of the prototypical basic amino acid-type NLS found in the SV40 large T antigen, only the second lysine in a continuous stretch of five basic amino acids is absolutely required for function (15). 

The NLS receptors for basic amino acid-type NLSs comprise a group of several proteins containing a central core of eight degenerate armadillo-type repeats of 42 amino acids flanked by N- and C-terminal nonrepeat regions (16). Although their precise role is unknown, the NLS binding site appears to occur within these multiple armadillo repeats.

The fact that the receptors for basic amino acid-type NLSs can functionally interact with a broad range of different sequences encouraged us to believe that chiral variants of this signal might be active in nuclear import. In this study, we have examined the effect of chirality on NLS function using an in vitro nuclear import assay with synthetic peptide conjugates. We have found that the basic amino acid-type NLS of the c-Myc protein is functional in both L- and D-forward and reverse configurations. Although the L configurations are proteolyzed in a cytosolic extract of cells, the D configuration is protease-resistant. This work presents results useful for functional studies of NLS-mediated transport and therapeutic applications and provides insight for understanding the nature of NLS ligand-receptor binding interactions.

**EXPERIMENTAL PROCEDURES**

**Preparation of Transport Substrate (FITC-BSA-NLS).**—The peptide-BSA conjugates used as transport substrates were prepared as described in Adam et al. (17) and Paschal and Gerace (18). HeLa cytosol was prepared as described by Adam et al. (17). The peptide-BSA conjugates used as transport substrates were prepared as described in Adam et al. (17). The activated BSA-FITC was mixed with 1 mg of either the L-forward, L-reverse, D-forward, or D-reverse NLS peptide and incubated overnight at 4°C. Peptide concentrations were determined by spectrophotometry absorbance at 205 and 214 nm and normalized. The recombinant import factors used to reconstitute import were 200 nm importin α, 200 nm importin β, 200 nm NTF2, and 1 μM Ran with 1% BSA in transport buffer.

**Nuclear Protein Import Assay.**—Nuclear protein import in digitonin-permeabilized HeLa cells was carried out as described by Adam et al. (17) and Paschal and Gerace (18). HeLa cytosol was prepared as described by Adam et al. (17). The peptide-BSA conjugates used as transport substrates were prepared as described in Adam et al. (17). The activated BSA-FITC was mixed with 1 mg of either the L-forward, L-reverse, D-forward, or D-reverse NLS peptide and incubated overnight at 4°C. Peptide concentrations were determined by spectrophotometry absorbance at 205 and 214 nm and added to BSA-FITC at normalized levels.

**Peptide Synthesis.**—Synthesis of peptides was carried out manually by established solid-phase synthesis protocol (19) on either 4-(hydroxymethyl)phenyl)-methyl or 4-aminomethyl-5-fluoropyrimidine (20) resins. Coupling was monitored by quantitative ninhydrin assay (21). The primary structure of each NLS peptide and that of SV40 L-forward, D-forward, L-reverse, and D-reverse c-Myc NLS peptide was dissolved in 100 μl of cytosol transport buffer (20 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 50 μl of this solution was mixed with 20 μl of purified HeLa cytosol, and the mixture was incubated at 37°C. Degradation of the NLS was monitored by analytical reversed phase HPLC with a gradient of 0–67% solution B in solution A over 30 min (solution A = 100% water, 0.1% trifluoroacetic acid; solution B = 90% acetonitrile, 10% water, 0.09% trifluoroacetic acid). Isolated peaks were analyzed by electrospray ionization mass spectrometry to determine their identity.

**RESULTS**

A schematic diagram depicting the chiral relationships between L- and D-forward and reverse peptides is shown in Fig. 1A. This figure illustrates the close structural similarity between the L-forward and D-reverse peptides. To investigate whether D-reverse peptides are biologically active as NLSs, we initially compared the nuclear import activity of a D-reverse peptide based on the SV40 T antigen NLS with its natural (L) counterpart (sequence shown in Fig. 1B). An L-reverse peptide based on the SV40 T antigen NLS has previously been shown to be largely inactive. For this analysis, fluorescent BSA-peptide conjugates were prepared and analyzed in a permeabilized cell assay for nuclear protein import. Although the wild type SV40 NLS peptide conjugate had the expected high level of nuclear import activity, the D-reverse peptide conjugate was essentially inactive (data not shown). We reasoned that the lack of activity in the D-reverse peptide conjugate could be due to the presence of a proline in the SV40 T antigen NLS. This is because proline, unlike other amino acids, forms a covalent bond with the main chain and is not topologically equivalent with respect to the N terminus in the forward and reverse directions. Most documented basic amino acid-type NLSs have a proline that closely flanks the core of basic residues (22), presumably to contribute to some contextual structure in the folded proteins. However, the c-Myc-NLS without its proline is known to have activity comparable with the SV40 signal (23, 24). Based upon these data, we decided to explore the chiral properties of the c-Myc NLS.

We synthesized the c-Myc NLS sequence in all four homochiral peptide orientations: all L-amino acids, all D-amino acids,
all L-amino acids synthesized in reverse, and all D-amino acids synthesized in reverse (Fig. 1A). These four peptides were coupled to FITC-conjugated BSA at an average ratio of five peptides/BSA molecule (as estimated by a size shift on SDS-polyacrylamide gel electrophoresis), and the conjugates (at 100 mM) were tested for their activity in the permeabilized cell import assay. To our surprise, all four c-Myc peptide-BSA conjugates were transported into the nucleus at similar levels as seen by fluorescence microscopy (Fig. 2A). The transport of these peptide conjugates occurred at a level comparable with a BSA conjugate containing the SV40 T antigen NLS coupled to BSA at a similar level (Fig. 2A). Quantitative analysis of the nuclear import of the c-Myc conjugates by flow cytometry (18) confirmed that all four conjugates were virtually identical in their transport activity and equivalent in activity to the SV40 T antigen conjugate (Fig. 2B). Control (not shown) experiments demonstrated that the nuclei of the permeabilized cells were almost all intact, as shown by their ability to exclude trypan blue (which is too large to diffuse into intact nuclei), and thus, the conjugates are unable to enter the nucleus by a nonphysiological diffusion route. Furthermore, nuclear accumulation of all four c-Myc conjugates was blocked by well characterized inhibitors of NLS-mediated nuclear import, including incubation at 0°C (L- and D-reverse conjugates shown in Fig. 2B; others not shown), depletion of ATP with hexokinase/glucose, and addition of GTPγS to the medium (data not shown). Together, these results indicate that the nuclear import of the c-Myc conjugates occurs by a physiological pathway (13).

We performed competition experiments to investigate whether these substrates enter the nucleus by the same pathway as the SV40 T antigen NLS. For this experiment, import assays were performed with wild type NLS-BSA conjugate in the presence of competing nonfluorescent NLS-BSA conjugates representing each chiral type at up to 50 μM as observed by microscopy (A). Competitions at 5, 10, and 30 μM were quantitatively measure by fluorescence-activated cell sorter (B). Dr and Lr, D and L reverse.

**Fig. 3.** All four chiral variants of the c-Myc peptide compete for a similar nuclear import pathway. Import assays were performed with wild type NLS-BSA conjugate in the presence of competing nonfluorescent NLS-BSA conjugates representing each chiral type at up to 50 μM as observed by microscopy (A). Competitions at 5, 10, and 30 μM were quantitatively measured by fluorescence-activated cell sorter (B). Dr and Lr, D and L reverse.
Note that the weaker activity of the L-reverse signal was not evident by fluorescence staining (Fig. 3A), which is included only to show a gross correspondence to the more sensitive fluorescence-activated cell sorter assay. To directly examine whether the transport competition by the c-Myc NLS peptide conjugates was due to binding to the NLS receptor, we carried out peptide cross-linking and competition experiments to measure the NLS receptor interaction of these peptides. Free unconjugated $^{125}$I-labeled SV40 T antigen NLS peptide was incubated with a cytosolic fraction enriched in the NLS receptor in the presence of a 200-fold excess of each of the four unlabelled c-Myc NLS peptides (Fig. 4), followed by cross-linking of the radiolabeled peptide to bound protein with 2,2-dimethyl-2-silapentanesulfonic acid and analysis of the products by SDS-polyacrylamide gel electrophoresis. As shown previously (14), we found that the SV40 T antigen NLS peptide was specifically cross-linked to the ~55-kDa NLS receptor in this assay (Fig. 4, lane 1) and was strongly competed by the homologous unlabelled NLS peptide (data not shown). Each of the free c-Myc peptides also competed with radiolabeled SV40 T antigen NLS for cross-linking to the NLS receptor (Fig. 4, lanes 2–3 and 4), although the L-reverse c-Myc peptide was somewhat less effective than the other three chiral versions (Fig. 4). To verify that the c-Myc NLS peptide conjugates utilizing the known basic-type NLS receptor pathway, all four conjugates were incubated with permeabilized cells supplemented solely with the recombinant import factors importin α, importin β, NTF2, and Ran. These factors alone were sufficient to reconstitute the import of the c-Myc NLS peptide conjugates in vitro (Fig. 2B).

To investigate the extent to which the D-forward and D-reverse peptides were proteolytically resistant in cytosol as compared with the L-peptides, we incubated each chiral variant of the c-Myc NLS as a free peptide in cytosol in the absence of protease inhibitors for varying times and analyzed the mixture by high performance liquid chromatography fractionation (Fig. 5). The peak corresponding to the L-peptide was degraded to a considerable extent by 15 min and was completely degraded at 4 h. By contrast, intact D-peptide was present at undiminished levels up to the longest time point tested (20 h). The high performance liquid chromatography fractions shown were taken at 1 min and 4 h for both the L-forward and D-reverse c-Myc. The later eluting L-forward peak is due to peptide dimer formation by a disulfide bond. This confirms that free L-forward peptides are quite susceptible to proteolytic degradation in cell cytosol. It also demonstrates that the D-peptide analogues of the c-Myc NLS would be useful as proteolytically resistant tools for therapeutic applications and for studies involving analysis of NLS function.

**DISCUSSION**

Our data indicate that all four chiral variants of the c-Myc NLS are equally active as nuclear import directing signals when analyzed as BSA-peptide conjugates in a permeabilized cell nuclear import assay. Furthermore, all four peptide-BSA conjugates compete for the import pathway used by the SV40 T antigen NLS in vitro as shown by substrate competition experiments, and all four variants specifically bind to the same NLS receptor as demonstrated by cross-linking competition. The observation that recombinant import factors alone are sufficient to direct the import of all four chiral conjugates in permeabilized cells supports the notion that these chiral NLS peptides interact with the same import machinery utilized by the SV40 NLS. The fact the importin α supported import of these conjugates, combined with our cross-linking data, strongly suggest that importin α is the receptor responsible for the observed import of the four chiral conjugates. Since the D-reverse and D-forward forms of the c-Myc NLS are functionally active (like the L-forward peptide) but are proteolytically resistant (unlike the L-peptide), these D-form NLSs will provide effective tools for analyzing the pathway of nuclear import in vivo and in vitro, where they could serve as stable competitive inhibitors. This is especially significant given the marked sensitivity to degradation we observed for the L-forward peptides. The D-form NLSs also could prove useful for gene delivery applications (26).

Although the L-reverse peptide conjugate of the c-Myc NLS was as active as the other three chiral forms in nuclear import, this peptide was somewhat less active than the other three chiral peptides in competing with the SV40 T antigen NLS peptide conjugate for import and for cross-linking to the NLS receptor. Among the four NLS variants, the D-forward peptide was most active and was the only one resistant to degradation.
receptor. It must be emphasized that each BSA molecule (on average) contained five NLS peptides, which were coupled to a heterogeneous range of sites that could influence the affinity of the immobilized NLS peptide for the NLS receptor (25). The rate of import could be determined by the highest affinity NLS site on a particular BSA molecule, whereas competition for import and peptide cross-linking would reflect an average affinity over the whole NLS population. It must be acknowledged that the diminished activity of the free L-reverse peptide during cross-linking could also be attributed to a lower resistance to proteolytic degradation.

Although there is no strict consensus sequence for basic-type NLS signals, there appear to be certain requirements that govern their activity. Previous work together with our own suggests that in addition to the need for basic amino acids, subtle structural requirements for activity must also be satisfied. In the case of the SV40 NLS, the presence of a Lys at position 128 is absolutely required for activity (15). An extensive study probing the functional requirements of this signal using conservative substitutions at position 128 revealed that a single β-Lys placed within the context of an otherwise L-NLS significantly reduced activity (27). In light of our own observation that an NLS peptide made entirely of D-amino acids is active, the disruptive effect of the above substitution suggests that the relative position of the charged side chains is important for activity and suggests that certain amino acid side chains must share the same relative orientation with respect to the main chain as their neighbors. Evidently, the activity of a NLS peptide depends less on absolute chirality than on the need for chiral uniformity.

Our study also suggests, in agreement with other work, that topology, rather than simply positive charge, plays a role in recognition of NLS signals. The widely reported inactivity of the reverse SV40 NLS and our observed inactivity of the D-reverse SV40 NLS suggests that the presence of a structurally incongruous proline overrides simple charge interactions. Supporting this notion is the observation that even the position of the proline in an all-L-forward NLS influences activity (28). Furthermore, we observed that a D-reverse SV40 NLS peptide with an alanine in place of the proline is an active NLS signal.3 Brady and Dodson (29) suggest that prolines, in particular, may present a major obstacle to the widespread application of D-reverse technology. Unlike all the other natural amino acids, the proline side chain forms a covalent bond with the main chain. The resulting structure is not topologically equivalent in the forward and reverse directions. Anchoring the side chain to the main chain restricts the angle φ to −60°, which favors the formation of β-turns, and for this reason, proline commonly occupies the i + 1 position in β turns. The presence of a proline may be particularly significant to the activity of NLS signals, since almost all identified basic-type NLS signals possess a flanking proline. Indeed, Chou Fasman predictions of 17 known nuclear and nucleolar sequences reveal a propensity to form turns (30). Crystallographic evidence that NLS sequences form turns via prolines can be seen in the 3-dimensional crystal structure of the human immunodeficiency virus matrix protein, where a proline plays a critical role in the formation of a loop structure that projects the NLS sequence above the plane of a surrounding β strand (31). Although free NLS peptides probably lack secondary structure, the restricted conformation of proline may impart some structural character to these peptides that influences receptor binding.

A rather general question arises from our study. How could the NLS receptor-mediated pathway recognize β-form peptides when they do not arise commonly in the intracellular environment? We suggest that the observed chiral promiscuity is a consequence of the fact that hundreds of different nuclear proteins must be accommodated by the NLS receptor-mediated import pathway. The NLS is usually located in an internal portion of the primary structure and must function without impinging upon the folding and functional requirements of the specific protein in which it resides. The compromise between the need for an easily recognized signal, and the need for that signal to be an integral part of structurally unrelated nuclear proteins necessitates the lack of consensus seen in NLS sequences. But this signal diversity presents a problem for receptor recognition. Since it would be too energetically expensive to produce a receptor that is tailor-made for each nuclear protein, we suggest that the signals are accommodated by a small group of fairly permissive receptors. Our study shows that this permissiveness extends to include signals whose chirality is entirely opposite to that of naturally occurring NLSs. By limiting signal recognition to a combination of charge and side chain topology, a few types of receptor could accommodate the plethora of structurally diverse proteins that must cross the nuclear envelope. Other intracellular signaling pathways that must process diverse proteins also exploit the use of signals with very loose consensus sequences, such as mitochondrial import (32), endoplasmic reticulum translocation (33), and lysosomal import pathways (34). Our study suggests that β-forward and D-reverse peptides may also be functional in these pathways, although these alternate chiral versions have yet to be tested. The structure of the NLS receptor co-crystallized with these chiral peptides should help to reconcile the apparent paradox of a structurally discerning, yet chirally promiscuous system.

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