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COMPARISON BETWEEN SYNTHETIC NUCLEAR LOCALIZATION SIGNAL PEPTIDES FROM THE STEROID/THYROID HORMONE RECEPTORS SUPERFAMILY

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ummary:The main objective of the study is to demonstrate that short basic peptides from the eroid/thyroid hormone receptors superfamily act as Nuclear Localization Signals out of receptors ontext. Such synthesized peptides, chemically coupled to Bovine Serum Albumin,were shown to enable ie corresponding BSA-conjugate to be transported to the nucleus. A second objective is to demonstrate ie utility of viral cointernalization as a good method for rapid quantitation, comparison and competition i nuclear entry.

It becomes now well-established that large nuclear proteins, which are synthesized in the cytoplasm, ise a molecular recognition step involving an intrinsic Nuclear Localization Signal (NLS) to enter the ucleus via nuclear pores. Though the identification of a "receptor" for such signals is an active field of esearch, it has not yet been precisely defined. The intrinsic signal, however, has been well-characterized n several nuclear proteins, the first one being the C-terminal region of nucleoplasmin (1). Other studies ave defined NLS as a short stretch of basic aminoacids such as the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val of SV40 large T antigen (2-5) though not all NLSs are of this type (6).

For about ten years, SV40-like NLSs have been characterized in many nuclear proteins but attempts o find a consensus, at least for sequence, have failed. In a way to make a good comparison in efficiency or nuclear targeting of several presumed SV40-like NLSs, we focussed the present study on epresentative members from a single nuclear protein family: the steroid/thyroid hormone receptors superfamily. Actually, since the question "*A nuclear localization signal in steroid hormone receptors ?*" was asked by Wolff et al. in 1987 (7), an answer is just beginning to raise from several studies (8,9). The problem of nuclear localization of members from the steroid/thyroid hormone receptor superfamily seems to be more complex than in the case of SV40 large T-antigen. Although all members share strong relatedness in structure and activity, it seems that the nuclear localization process is depending on the considered receptor: some, like the glucocorticoid receptor are cytoplasmic in the absence of ligand and become nuclear in its presence (10); others, like the estrogen receptor (11) or the retinoic acid receptors (12) are nuclear, even when unliganded.

Many studies at the moment are aimed to explain these differences in nuclear localization regulation. Results agree to define a primary SV40-related NLS in all receptors between the DNA- and hormone-binding regions. However, different regulated transports put forward the presence of a second NLS (9)

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except for the œstrogen receptor (13) or dimerization (7) and more recently, the presence of an intrins "inactivation function" in certain cases (12).

Indeed, problems of nuclear transport regulation of these hormone-inducible transcription factors a quite essential to elucidate for a good understanding of signal transduction by steroid hormones. By tl present study, we would like to settle up with the primary SV40-related NLSs of receptors which a representative of the superfamily as to bring a contribution to further works concerning the regulation(s)

There are different ways to demonstrate a peptidic sequence to be a nuclear localization signal: tl presumed NLS region of the nuclear protein can be deleted or mutated in as to show its essential natur other assays demonstrating that the NLS is sufficient for nuclear targeting use either molecular biolo techniques to obtain fusion proteins or chemical crosslinking of the synthetic NLS to a nonnucle protein. In this study, we used this latter technique as to be able to compare different receptor NLSs o of the receptor context and consequent regulation. We crosslinked Bovine Serum Albumin (BSA) wi peptides corresponding to the presumed NLSs from human Retinoic Acid Receptors (hRARs), huma Glucocorticoid Receptor (hGR), human Estrogen Receptor (hER), human Androgen Receptor (hAR) ar SV40 large T-antigen as a positive control. For introducing the NLS-BSA complexes in the cytoplasm c cultured cells, we chose to make use of viral cointernalization, an original method which we have recentl described to be useful for this purpose (14).

MATERIALS AND METHODS

Peptide synthesis: The peptides were synthesized on a LKB Biolynx peptide synthesizer usin Fmoc-amino acids chemistry (15). After cleavage from the resin and deprotection using aqueou trifluoroacetic acid, peptides were purified by high performance liquid chromatography on a C18 columr eluted by 20% aqueous acetonitrile(Yield 79-91%). Purity was assessed by analytical reversed-phase high-pressure liquid chromatography, aminoacid analysis, and fast atom bombardment-mas spectroscopy (see Table I).

Peptide-protein conjugation: The synthetic peptides were conjugated to bovine serum albumin (BSA with a bifunctional cross-linking reagent, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS according to Lerner (16).

A sample was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5-30%) tc visualize an increase in molecular weight due to the addition of peptide to the protein. A more accurate estimate of the average degree of conjugation was determined by amino-acid analysis (3 measurements).

Viral co-internalization: The adenovirus serotype 3 (subgroup B) was produced in suspension grown KB cells. Adenovirons were extracted and purified in CsCl gradients, as previously described (17) and the virus titers were determined and expressed as fluorescent focus units (FFU). MCF 7 and HeLa S3 cells monolayers were infected with adenovirus 3 suspensions (300FFU/cell) in Dulbeco medium including BSA or peptide-BSA at 1 mg/ml. After 45 minutes incubation at 37°C (kinetic determined in (13)), cells were prepared for immunofluorescence.

Immunofluorescence: Soon after incubation cells were harvested for immunofluorescence according to Langranger et al. (18). BSA was detected by indirect immunofluorescence using goat anti BSA IgG (Sigma, St Louis, USA). Internalized fluorescent macromolecules were examined in an Olympus BH-2 microscope (excitation wavelength at 530-560nm, edge filter at 580nm). Photomicrographs were taken using a Kodak citachrome film (400 Asa).

RESULTS

Conjugation of the synthetic peptides to a carrier protein:.Synthetic peptides were synthesized which contain a 7-9 amino acids core sequence supposed to act as nuclear localization signal and the sequence Cys-Gly-Tyr-Gly was added to the amino terminal as a spacer containing a cysteinyl residue for ease of coupling to a carrier protein (5). They were coupled to bovine serum albumin (BSA) chosen as carrier

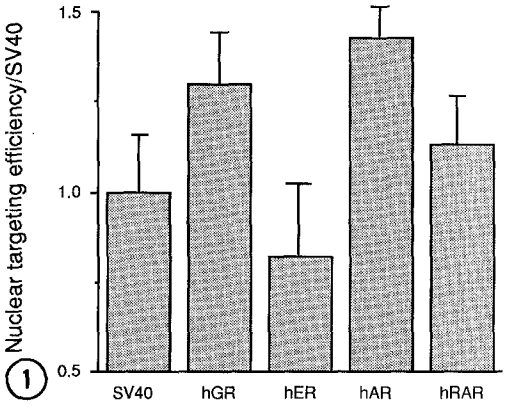
rotein because of its widespread use in similar studies. Coupling was made using the m-naleimidobenzoyl-N-hydroxysuccinimide ester (MBS) procedure (16). The coupling ratios were stimated by two methods : estimation of the increase in apparent molecular weight of BSA by mobility etardation by sodium dodecyl sulfate polyacrylamide gel electrophoresis and amino acid analysis. The ncrease in the ratios of residues contained in each complex are reported in table I.

Comparison of nuclear targeting efficiencies:.Soon after Adenovirus infection in the presence of 3SA or peptide-BSA, cells were returned to a CO_2 incubator for 45 minutes and then immediately arvested for immunofluorescence. Microphotographs were taken with the concern of keeping the same onditions: microscope enlargment, film, aperture and exposure time. Negatives were scanned using a oyce-Loeble micro-densitometer. For each assay 20 cells were scanned horizontally following the ongest line passing through the nucleus, area under the optic density curve was integrated and a nuclear mmunofluorescence/cytoplasmic fluorescence value was averaged. The values for each complex were educed to the corresponding coupling ratios and the nuclear targeting efficiency of SV40 fixed to 1 as to ompare with literature data. The other values were then calculated to give for each one a nuclear argeting efficiency/SV40 reported on histogram in fig 1. Results reported indicate that:(i) all presumed NLSs are efficient for nuclear transport, (ii) efficiency values are in the range of the one for SV40 large T antigen, and (iii) there are sensible differences for these values between receptors (e.g 1:2 for hRAR and hGR).

Competition with free peptide: A single competition experiment is described for hRARNLS-BSA complex versus free hRARNLS, this choice will be discussed further. For assay 0.5, 1, 2, 5 or 10 equivalents of free peptide were co-added with the complex in the medium. An equivalent is calculated taking into account the concentration of the complex multiplied by the coupling ratio. Figure 2 shows a graph in which, for each point, nuclear immunofluorescence/cytoplasmic immunofluorescence ratio was calculated as previously described except that the values were reduced to fix immunofluorescence of complex hRARNLS-BSA complex alone to 100%. Results indicate that hRAR-NLS is a good competitor in the conditions used in this work.

Table 1

Peptide	Sequence	FAB M+	Amino acid analysis	PAGE av.MW	Coupling ratio
SV40	CGYGPKKKRKV	1263.6	Gly: 1.81 Cys: 0.89 Tyr: 1.18 Pro: 1.15 Arg: 1.15 Lys: 4.125 Val: 1.16	74-76,000	5.1
hRAR	CGYGDRNKKKKE	1310.4	Gly: 2.06 Cys: 1.68 Tyr: 1.035 Asx: 1.88 Arg: 1.02 Lys: 4.02 Glu: 0.96	72-74,000	4.2
hGR	CGYGARKTKKKIK	1480.9	Gly: 2.12 Cys: 1.18 Tyr: 1.01 Ala: 1.17 Arg: 1.09 Lys: 4.81 Thr: 1.17 Ile: 1.04	74-76,000	4.3
hER	CGYGIRKDRRGGR	1493.8	Gly: 3.98 Cys: 1.20 Tyr: 0.96 Asx: 0.94 Arg: 4.11 Lys: 1.00 Ile: 1.12	75-77,000	4.6
hAR	CGYGARKLKKLGN	1407.8	Gly: 1.88 Cys: 1.07 Tyr: 0.88 Asx: 0.92 Arg:1.01 Lys: 3.31 Ala: 1.00 Leu: 2.07	72-74,000	4.2



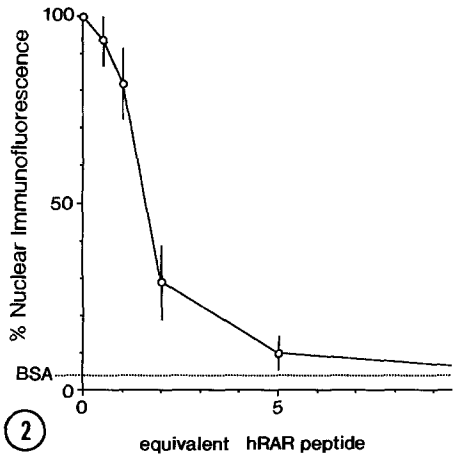


Figure 1. Comparison between nuclear targeting efficiencies of nuclear localization signal peptides. Values are calculated as indicated in materials and methods.

Figure 2. Competition for nuclear entry between hRAR-NLS-BSA complex and free hRAR-NLS.Calculations for percentage of nuclear immunofluorescence and equivalent of competitor peptide are described in the text.

DISCUSSION

The work presented here clearly demonstrates that SV40 T-antigen related NLS-sequences of four receptors from the steroid/thyroid hormone receptors superfamily can target a normally nonnuclear protein to the nuclear compartment. The essential character for nuclear entry was already demonstrated for two of them by mutation studies (8,9) while for other members this role was inferential. However this is the first time to our knowledge that the sufficient nature for nuclear targeting of such peptides, is reported out of the receptor context. Though there are sensible differences between evaluated nuclear targeting efficiencies, no relationship could be made with regulation of nuclear transport for the different receptors.

The competition study was organized for a single peptide hRAR, and this for at least two reasons; (i) we would like to verify if this kind of assay, already described using micro-injection (19), could be applied to the viral co-internalization method, and (ii) retinoic acid receptor is peculiar regarding all other members of the superfamily because in a recent report, Robbins et al. (20) put forward the presence of two basic aminoacids ten residues before presumed NLSs in number of nuclear proteins. These are conserved too throughout the superfamily except for retinoic acid receptors. However hRAR-NLS seems to be as efficient as other NLSs and the question of the role played by the short conserved basic stretch still remains.

In the view of the results presented here, one cannot argue that SV40-like primary NLSs detected in the steroid/thyroid hormone receptors superfamily are the only functional signal within the protein. However, in the limits of immunofluorescence detection, accumulation seems to be complete in our experiments in contrast to the work presented by Robbins et al. (20) on nucleoplasmin. This does not exclude further studies to be conducted in order to see the influence of other parts of the protein, in particular the N-terminal part of the putative bipartite signal, since this motif is present in many receptors of the superfamily.

Results from the competition experiment show that, in the conditions used here (45 minutes ıcubation time), hRAR-NLS alone is able to dramatically decrease nuclear entry of the hRAR-NLS-SA complex at ratios higher than 5 equivalents. At least, two explanations can be put forward for this ıther high value: (i) the effect of position and number of NLSs seem to play an important role on ansport (5) and (ii) it has been shown that competition essentially affects the rate of nuclear ɔcumulation (19).

The peptides studied here were chosen to be heterogen, at least in sequence, and thus to be ɛpresentative for the numerous members of the superfamily. Residues contained in the sequences are, ɔr the majority, positively charged at physiological pH. These positive charges are not equally istributed in each peptide except for the presence of one basic aminoacid at a position that we have ıade to coincide with the first lysine of SV40 T-antigen. Actually, this position corresponds to the rucial Lys_{128} in SV40 T-antigen which can be replaced (keeping nuclear targeting activity but less fficiency) by any positive charged group (21). Except for a basic residue at this position, we failed in efining a precise consensus because the studied peptides are all active for nuclear localization with ıositive charges unequally distributed for each. However, our results are in accordance with the videspread hypothesis that nuclear uptake is a receptor-mediated process involving semi-specific nteractions. Works are in progress, taking into account the structural organization of such NLSs, in a vay to establish a 3-dimensional map of charges repartition which should be important in the protein-ırotein interaction step that probably triggers the nuclear entry of macromolecules. Designing a simple ɔnsensual 3D-structure efficient for karyophylic function should be of utmost interest in targeting ɛnobiotics to the nucleus for instance.

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