J. Steroid Biochem. Molec. Biol. Vol. 40, No. 1–3, pp. 279–285, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

NUCLEAR AND NUCLEAR ENVELOPE BINDING PROTEINS OF THE GLUCOCORTICOID RECEPTOR NUCLEAR LOCALIZATION PEPTIDE IDENTIFIED BY CROSSLINKING

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Summary—The molecular mechanisms underlying the nuclear entry of steroid receptors and possible regulation of steroid hormone action during receptor passage across the nuclear envelope have not been elucidated. A nuclear localization signal has been identified in the hinge region of the glucocorticoid receptor. A synthetic peptide corresponding to this sequence was radio-iodinated and incubated with high salt- and detergent-extracted rat liver nuclei or nuclear envelope in the presence of crosslinker. After SDS–PAGE, two nuclear polypeptides of 60 and 76 kDa which had been specifically crosslinked were identified by autoradiography. A 60 kDa polypeptide was also crosslinked in the nuclear envelope fraction. ATP and elevated temperatures enhanced the crosslinking of both nuclear peptides. Finally, we showed that the pattern of crosslinking of the simian virus 40 large tumour antigen nuclear localization signal was identical to that of the glucocorticoid receptor signal to the nuclear polypeptides. The crosslinked peptides are good candidates for nuclear importers of the glucocorticoid receptor. In addition, the data suggest that these binding sites may be part of a general mechanism for nuclear entry of proteins.

INTRODUCTION

Little is known about the molecular mechanisms involved in the import of protein into the nucleus and possible mechanisms of regulation of entry into the nucleus. This lack of knowledge extends to the mode of nuclear entry of steroid receptors and modulation of steroid hormone action during receptor passage across the nuclear envelope. In the absence of steroid, cellular glucocorticoid receptor is predominantly cytoplasmic [1–3]. Only upon binding of glucocorticoid does the glucocorticoid receptor translocate to the nucleus [4, 5]. We have previously shown the association of the glucocorticoid receptor with rat liver nuclear envelope using immunocytochemistry [6], binding studies [7], immunoblotting and affinity labelling [8]. Significantly, the immunoblot analysis revealed immunoreactive glucocorticoid receptor in extracts from nuclear envelope isolated from intact, but not from adrenalectomized rats. These data show that in the presence of glucocorticoid, part of the cellular glucocorticoid receptor is localized to the nuclear envelope. A reasonable speculation is that this receptor is interacting with transport machinery located on this membrane barrier.

A nuclear localization signal in the hinge region of the glucocorticoid receptor has been identified by the present authors (TKKKIKG; residues 493–499 of the human glucocorticoid receptor; residues 512–518 of the rat glucocorticoid receptor) and others (RKTKKKIK; residues 491–498) [9] by its homology with the SV40 large-T antigen nuclear localization signal (PKKKRKV) [10–12] and by deletion mutation experiments of the rat glucocorticoid receptor cDNA [5]. A similar sequence has been defined by deletional analysis of the rabbit progesterone receptor cDNA [13]. In this paper we report the identification of nuclear and nuclear envelope peptides which interact with the nuclear localization signal, achieved by crosslinking radio-iodinated synthetic peptide corresponding to the signal to high salt- and detergent-extracted rat liver nuclei or nuclear envelopes.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats weighing 200–250 g were obtained from Charles River

Proceedings of the VIIIth International Congress on Hormonal Steroids, The Hague, The Netherlands. 16–21 September 1990.

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Canada Inc. and maintained on a diet of Purina lab chow and tap water *ad libitum*. The rats were sacrificed by decapitation and the livers were quickly removed and placed in ice-cold homogenization buffer (0.32 M sucrose containing 3 mM $MgCl_2$ and 1 mM DTT). The livers were rapidly stripped of connective tissue, weighed and minced.

Peptide synthesis

The putative nuclear localization signal identified in the glucocorticoid receptor was commercially synthesized by IAF BioChem Int. Inc. The final product was: H-Cys-Gly-Tyr-Gly-Thr-Lys-Lys-Lys-Ile-Lys-Gly-OH (signal underlined) with $M_W = 1{,}182$.

Purification of nuclei and nuclear envelopes

The preparation of nuclei and nuclear envelopes has been described previously [7]. Nuclei and nuclear envelopes used in crosslinking studies were resuspended in crosslinking buffer (0.25 M sucrose, 10 mM HEPES–KOH, pH 7.4, 25 mM KCl, 3 mM $MgCl_2$, 5 mM NaI) and stored at −75°C after rapid freezing in liquid nitrogen.

Radio-iodination of synthetic peptide

The peptides were radiolabelled using IODOGEN™ (Pierce Chemical Co.) [14]. Unincorporated $Na^{125}I$ was removed by spin filtration at 1600 *g* for 4 min through a 1 ml column of Sephadex G-10 (Pharmacia), equilibrated in crosslinking buffer. Iodination yielded sp. act. = 1.3–2.0 × 10^6 cpm/μg peptide, assuming 100% recovery of peptide.

Crosslinking studies

Nuclei or nuclear envelopes (50–100 μg protein) in as small a volume as possible were incubated with an equal volume of 2% octyl-β-D-thioglucopyranoside (Calbiochem) and 600 mM KCl for 1 h on ice. The extracts were diluted 6-fold in crosslinking buffer, approx. 1 × 10^6 cpm of I^{125}-labelled nuclear localization signal peptide (approx. 0.7 μg protein) was added with or without a 100-fold excess of cold competing peptide, and incubated for 30 min at room temperature. The samples were then cooled on ice for 5 min. Crosslinker, bis(sulfosuccinimidyl)suberate (Pierce Chemical Co.), was added to a final concentration of 0.09, 0.50 or 1.0 mM. Crosslinking at room temperature was terminated after 15–60 min by dilution to a final vol of 1 ml and precipitation with 10% trichloroacetic acid on ice for at least 5 min. The precipitates were pelleted by microcentrifugation for 3 min, then washed twice with 95% acetone, air-dried before resuspension in 2 × sample buffer [15] and heat denatured for 3 min at 100°C. Samples were run on SDS–PAGE, stained with Coomassie blue and dried before autoradiography with an intensifying screen (Dupont Cronex, Lightning-Plus™) at −75°C.

Protein determination and gel electrophoresis

The protein content was determined by the method of Lowry *et al.* [16], using BSA as a standard. SDS–PAGE was performed using the discontinuous buffer system of Laemmli [15].

RESULTS

Nuclear localization signals

A seven amino acid sequence of the SV40 large-T antigen has been shown to act as an autonomous signal capable of specifying nuclear localization of non-nuclear proteins [10, 11]. We subjected this heptamer to alignment analysis with the amino acid sequences deduced from steroid receptor cDNAs. The search was set to detect a minimum of three identical amino acids while maintaining at least 50% of the charge identity. The alignment analysis was performed visually and confirmed using the Microgenie™ program (Beckman). Strong homology with the SV40 large-T antigen nuclear localization sequence was identified in the androgen [17, 18], estrogen [19], glucocorticoid [20], mineralocorticoid [21] and progesterone receptors [22] (Table 1). Sequence homologies to the heptamer were also found within the amino acid sequence of other members of the steroid receptor superfamily [23], including the c-*erb*A/thyroid hormone receptors [24–26], retinoic acid receptors [27–29] and the vitamin D receptor [30] (Table 1). The putative nuclear localization sequence lies in the hinge region of these DNA-binding proteins, between the carboxy-terminus of the DNA-binding domain of the receptor and the amino-terminus of the hormone-binding domain. However, a newly discovered member of the steroid receptor superfamily called COUP-TF [31] lacks any homology with the signal in the hinge region. Unlike most steroid receptors, COUP-TF does not exceed the diffusion limit of the nuclear pore complex [32], the supposed

Table 1. Potential nuclear localization sequences in steroid and nuclear hormone receptors

	Sequence	Ref.
SV40 large-T antigen	^{126}PKKKRKV^{132}	[12]
Receptor		
Human glucocorticoid	^{493}TKKKIKG^{499}	[20]
Human mineralocorticoid	^{675}SKKLGKL^{681}	[21]
Human progesterone	^{639}FKKFNKV^{645}	[22]
Human estrogen	^{263}RMLKHKR^{269} ^{298}IKRSKKN^{304}	[19]
Human androgen	$^{627/628}$ARKLKKL$^{633/634}$	[17, 18]
Human thyroid hormone		
Type α	^{132}VAKRKLI^{138}	[24, 25]
Type β	^{181}LAKRKLI^{187}	[26]
Human vitamin D	^{106}MILKRKE^{112}	[30]
Human retinoic acid		
Type α	^{162}RNKKKKE^{168}	[27]
Type β (hap)	^{155}RNKKKKE^{161}	[28, 29]

mediator of nucleocytoplasmic exchange [reviewed in 32–34]. Therefore COUP-TF may not require a nuclear localization signal. Alternatively, the sequence may be elsewhere in the molecule.

The nuclear localization signal that was identified in the steroid receptor superfamily was only found in those steroid binding proteins which act within the cell nucleus. The sequence was not present in human corticosteroid binding globulin [35], human sex hormone-binding globulin [36] and rat androgen-binding protein [37].

Crosslinking studies

Detergent- and high salt-extracted nuclei and nuclear envelopes were incubated with radioiodinated glucocorticoid receptor nuclear localization peptide with or without a 100-fold molar excess of cold peptide in the presence of crosslinker. The crosslinked products were separated on SDS–PAGE and subjected to autoradiography. Figure 1 shows that two nuclear peptides of relative M_W 60,000 and 76,000 were specifically crosslinked (this includes the molecular weight of the crosslinked peptide). In the nuclear envelope fraction, we consistently have observed a 60 kDA crosslinked product. However, the 76 kDa crosslinked peptide is not always observed in this sub-nuclear fraction.

Imamoto-Sonobe *et al.* [38] have shown that the efficient association of nuclear proteins with isolated rat liver nuclei requires ATP and that the association occurred at 33°C but not at 4°C. Other groups have shown nuclear import to be a two-step process [39, 40]. The first step, binding of the protein to the nuclear envelope, was shown to be independent of ATP, while the second step, import of the protein into the nucleus, required ATP. We investigated the

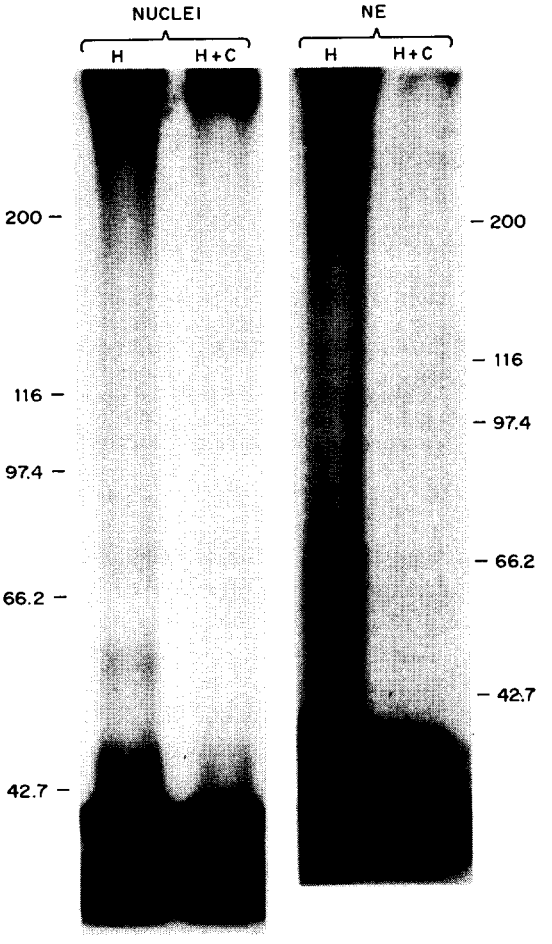

Fig. 1. Identification of nuclear and nuclear envelope polypeptides crosslinked to radioiodinated nuclear localization signal of the glucocorticoid receptor. Nuclei (50 μg protein) or nuclear envelopes (NE; 100 μg protein) were incubated with 1.6 and 0.3 μM, respectively, of ^{125}I-labelled signal peptide and 1.0 and 0.09 mM, respectively, crosslinker in the presence (H + C) and absence (H) of 100-fold molar excess unlabelled signal and run on SDS–PAGE before autoradiography as described in the Experimental section. Molecular weight standards correspond to myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (97,400), BSA (66,200) and ovalbumin (42,699).

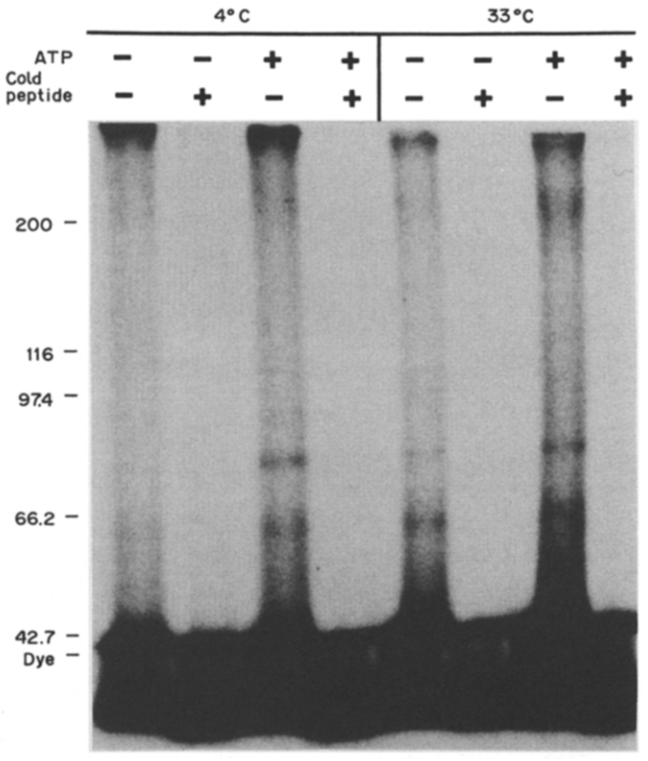

Fig. 2. The effect of temperature and ATP on the crosslinking of nuclear polypeptides to the nuclear localization signal of the glucocorticoid receptor. Nuclei (100 μg protein) were pre-incubated for 2 h at 33°C in 10 mM HEPES (pH 7.8), 60 mM KCl, 2 mM $CaCl_2$, 3 mM $NaHCO_3$, with or without 0.4 mM ATP. Nuclei were then extracted with 1% octyl-β-D-thio-glucopyranoside/300 mM KCl for 1 h at 4°C and incubated with 1.1 μM ^{125}I-labelled signal peptide and 0.09 mM crosslinker for 60 min in the presence or absence of 1 mM ATP either at 4°C or 33°C. Crosslinking was performed at room temperature in the presence (H + C) or absence (H) of 100-fold excess of unlabelled peptide, before running on SDS–PAGE and autoradiography.

energy requirements in the crosslinking studies of the nuclear fraction (Fig. 2). At 4°C, in the absence of ATP, a faint band was observed at 60 kDa. After addition of ATP at this temperature, the intensity of this band increased and a distinct crosslinked product at 76 kDa was observed. At 33°C, in the absence of ATP both bands were visible, but the intensity of each was increased after incubation in ATP.

We compared the profile of crosslinked peptides obtained after incubation of nuclei with radio-iodinated glucocorticoid receptor nuclear localization peptide with that obtained after nuclei had been incubated with radio-iodinated SV40 large T-antigen nuclear localization peptide. Significantly, Fig. 3 shows that the patterns were identical.

DISCUSSION

We have identified androgen-binding sites on rat ventral prostate nuclear envelopes which resemble the nuclear androgen receptor [41] and a population of glucocorticoid receptors resident on the rat liver nuclear envelopes [6–8]. It has been our hypothesis that these receptors are in the process of translocation across the nuclear envelope. This hypothesis has been strengthened by electron microscopic studies in which the nuclear envelope glucocorticoid receptors have been localized to the nuclear pore complexes [6]. As regulators of gene expression such as steroid receptors must traverse the nuclear envelope and enter the nucleus to effect their action, the elucidation of the mechanism by which they are transported across the nuclear envelope is critical to our understanding of cellular processes. This has been highlighted by recent evidence for an active role of nucleocytoplasmic exchange in regulation of cell function. A gradient in the nuclear localization of a candidate protein morphogen in the developing *Drosophila* embryo has been identified [42, 43]. The morphogen is active only when in the nucleus and nuclear localization was established to be temporally regulated.

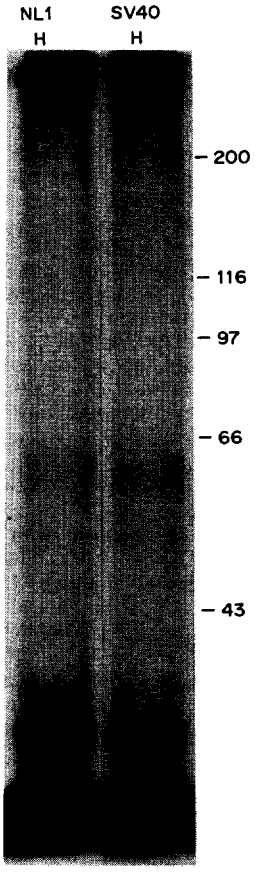

Fig. 3. Comparison of nuclear polypeptides crosslinked to the radio-iodinated nuclear localization signal of the glucocorticoid receptor and the nuclear localization signal of the SV40 large-T antigen. Liver nuclei (50 μg protein) were incubated with ^{125}I-labelled glucocorticoid receptor signal (NL1; 0.5 μM) or large-T antigen signal (SV40; 0.5 μM) and run on SDS-PAGE before autoradiography as described in the Experimental section, except that there was no trichloroacetic acid precipitation. The volume of the crosslinking reaction was kept to 100 μl and the reaction was terminated by adding an equal volume of 2 × sample buffer and then applying directly to the gel.

Such a gradient may be regulated by: (1) anchoring of the molecule in the cytoplasm to other proteins, such as heat shock protein 90 in the case of steroid receptors [44], where binding of the ligand to the receptor could release it from its anchor [45] or covalent modification of the anchor protein or the anchored protein could cause a change in subcellular distribution; and (2) controlled passage across the nuclear envelope.

Controlled passage of large proteins across the nuclear envelope, by analogy with mitochondrial and endoplasmic proteins, requires: (1) a signal for nuclear migration within the protein itself; and (2) a mechanism at the nucleus to respond to the signal. In this paper we have begun to delineate the second of these requirements. We have shown that the nuclear localization signal we identified interacts specifically with two nuclear peptides of 60 and 76 kDa. A 60 kDa peptide was also identified on the nuclear envelope and it is reasonable to assume that this is the same as that identified in the nuclear fraction. The pattern of crosslinking of the nuclear fraction with the SV40 large-T antigen nuclear localization signal was identical suggesting that as in mitochondrial protein import, a general import protein exists. In support of this, a common component in all systems used to identify binding proteins for various nuclear localization signals in both rat liver cells [46–49] and yeast [50, 51] is a protein of approx. 60 kDa. Another common element is a protein of approx. 70 kDa [46–51]. We see a higher molecular weight species of 76 kDa, whereas only one other group observed a binding protein of similar weight [49]. This protein may be a protein more specific for the transported molecule.

A 28 amino acid region encompassing a similar sequence to the SV40 large-T antigen nuclear localization signal has been identified by Picard and Yamamoto [5] in the glucocorticoid receptor and a similar region has also been identified as a nuclear localization signal in the rabbit progesterone receptor [13]. Others [9, 13] have identified slightly different sequences of the glucocorticoid receptor as homologous to the SV40 large-T antigen nuclear localization signal that we have identified. This is because two possible alignments exist. In fact, in the estrogen receptor three different sequences (ours and Refs [9, 13]) are possible. It is possible that all or only selected sequences act as nuclear localization signals. In fact, Picard *et al.* [52] have recently identified by deletional analysis a region of the human estrogen receptor, containing these three different sequences (amino acids 256–303), as the nuclear localization signal sequence. The sequence 263–271 alone failed to direct a fusion protein to the nucleus. According to their results, none of the basic stretches is in itself sufficient. The two extra basic residues identified by Wolff *et al.* [9] and Guiochon-Mantel *et al.* [13] might increase the effectiveness of the nuclear localization signal used in this study. We have identified a minimum nuclear localization signal which may be dependent on the protein context in which it is present, e.g. stabilization of the secondary structure by flanking sequences, modulation of activity by post-translational modification of its sequence and

flanking sequences or masking of the sequence by tertiary or quaternary structure of the protein.

It is possible from our data and that of others on the transformation of steroid receptors to design a model for further investigation in which after the nuclear localization signal of the glucocorticoid receptor is unmasked by binding of the glucocorticoid to its receptor [45], the signal is able to bind to a general nuclear envelope import or docking protein of 60 kDa. Thereafter it is able to bind to a 76 kDa nuclear importer peptide and gain access to the intranuclear compartment. In addition a second nuclear localization signal has been identified in the steroid binding domain of the glucocorticoid receptor [5]. The role of the other nuclear localization sequences in relation to the nuclear localization signal used in these studies requires investigation.

Acknowledgements—This investigation was funded by the Medical Research Council of Canada. E. C. LaCasse is the recipient of a studentship from the 'Fonds pour la Formation de Chercheurs et l'Aide à la Recherche' of Quebec.

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