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The Interdomain Region of Dengue NS5 Protein That Binds to the Viral Helicase NS3 Contains Independently Functional Importin β1 and Importin α/β-Recognized Nuclear Localization Signals*

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Dengue virus NS5 protein is a multifunctional RNA-dependent RNA polymerase that is essential for virus replication. We have shown previously that the 37-amino acid interdomain spacer sequence (residues $^{369}X_2$KKX_{14}KKKX_{11}RK$X_3{}^{405}$) of Dengue2 NS5 contains a functional nuclear localization signal (NLS). In this study, β-galactosidase fusion proteins carrying point mutations of the positively charged residues or truncations of the interdomain linker region (residues 369–389 or residues 386–405) were analyzed for nuclear import and importin binding activities to show that the N-terminal part of the linker region (residues 369–389, a/bNLS) is critical for nuclear localization and is recognized with high affinity by the conventional NLS-binding importin α/β heterodimeric nuclear import receptor. We also show that the importin β-binding site (residues 320–368, bNLS) adjacent to the a/bNLS, previously identified by yeast two-hybrid analysis, is functional as an NLS, recognized with high affinity by importin β, and able to target β-galactosidase to the nucleus. Intriguingly, the bNLS is highly conserved among Dengue and related flaviviruses, implying a general role for the region and importin β in the infectious cycle.

Dengue virus is a member of the genus *Flavivirus* within the family Flaviviridae that also contains the genera *Pestivirus* and *Hepacivirus*. There are around 70 viruses grouped in the *Flavivirus* genus, which includes yellow fever virus (YFV),[1] Japanese encephalitis virus, Murray Valley encephalitis virus, Kunjin virus, and tick-borne encephalitis virus. Dengue virus causes a benign syndrome known as Dengue fever and a more severe illness, Dengue hemorrhagic fever or in its severest form Dengue shock syndrome (1–3). There are four serologically and phylogenetically distinguishable Dengue viruses (types 1–4), and the disease they cause is of substantial world wide significance to human health but is mostly restricted to tropical and sub-tropical areas because of its transmission by the *Aedes aegypti* mosquito (4).

Flaviviruses possess a single-strand, positive-sense RNA genome of around 11 kb, which is capped but not polyadenylated, and encodes a single polyprotein including three structural and seven non-structural proteins in the order C-prM-E-NS1-NS2A-NS3-NS2B-NS2A-NS4B-NS5 (5, 6). Replication of flaviviruses occurs at membrane-associated replicase complexes localized in the perinuclear region. The replicase complex has been extensively characterized in several flaviviruses and includes NS1, NS2A, NS3, NS4A, and NS5 (7–12) and possibly some cellular proteins (13–15). Whereas the protein and RNA interactions of the replicase complexes still require detailed characterization, several recent studies (7, 17) have focused on the structure and function of the NS3 and NS5 proteins.

NS3 (69 kDa) is a multifunctional protein that has been shown to have protease, helicase, NTPase, and 5′-terminal RNA triphosphatase activities (16–19). NS5 (104 kDa) contains a well characterized RNA-dependent RNA polymerase activity associated with the C-terminal domain (20–24). Although there has been no function demonstrated for the N-terminal domain of NS5, it is predicted to be an *S*-adenosylmethionine transferase based on sequence comparison (25).

Whereas the primary replication activity involving Dengue NS5 occurs in the cytoplasm, a predominantly nuclear hyperphosphorylated form of NS5 has been reported in infected mammalian cells in the late stage of infection. The cytoplasmic form of NS5 is hypophosphorylated and able to interact with NS3, whereas the hyperphosphorylated form does not interact with NS3 (7). Phosphorylation of flavivirus NS5/NS5A proteins occurs at serine/threonine residues (26, 27). YFV NS5 is also phosphorylated and localized in the nucleus (28), whereas NS5 of other flaviviruses such as Japanese encephalitis virus and Kunjin virus have not been detected in the nucleus (8, 29).

Transport to the nucleus of proteins that are larger than 45 kDa requires intrinsic targeting signals called nuclear localization sequences (NLSs) (30–33). There are two main classes of basic type NLSs as follows: the simian virus 40 (SV40) large tumor antigen (T-ag) monopartite NLS type, consisting of a single cluster of basic amino acids (PKKKRKV) (34); and bipartite NLSs comprising two clusters of basic amino acids separated by a spacer of 10–12 amino acids (35). Both types of NLS are recognized by the conventional nuclear import receptor, the importin α/β heterodimer (36–39), which mediates binding to the nuclear envelope, translocation through it, and release within the nucleus, in conjunction with other compo-

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[1] The abbreviations used are: YFV, yellow fever virus; NLS, nuclear localization signal; β-gal, β-galactosidase; T-ag, T-antigen; GST, glutathione *S*-transferase; ELISA, enzyme-linked immunosorbent assay; CLSM, confocal laser scanning microscopy; Y2H, yeast two-hybrid; IBB, importin β binding.

TABLE I
Primers used to generate NS5 fusion protein expression constructs

Primer names indicate regions targeted, the orientation (in parentheses), and restriction sites used. Underlined nucleotides within primers indicate restriction sites, and nucleotides in bold denote introduced mutations. The general primers (d) have been described previously (44).

(a) Primers for Mutagensis	Sequence (5'→3')	
NLS (Rvs) R401N K402N *Bam*HI	GACGGGATCCGAGCTTCTC**T**CA**TT**A**TT**TGTGAATTCTT	- sense
NLS (Fwd) K371N K372N *Nco*I	CGCCCGCCATGGGCACAAA**T**AA**T**CTAATGAAAATCACG	+ sense
NLS (Rvs) K388N K389N *Avr*II	GTGCACATCCTAGGTGTA**TT**ATTCTTTCCTAG	- sense
(b) Primers for NS5-bNLS		
bNLS (320) (Fwd) *Nco*I	TCAGCATCATCCATGGTGAA	+ sense
bNLS (368) (Rvs) *Bam*HI	TTTTGGATCCGATTCTTTCGGTTCTTGGGTTCTCGTGTC	- sense
NS5-341 (Fwd) *Nco*I	ACACCCATGGCAATGACAGACACGACTCC	+ sense
(c) Primer for NS5 (386-405)		
NS5 (386) (Fwd) *Nco*I	GAAAGCCATGGGAAAGAAAAAGACACCTAG	+ sense
(d) General Primers		
NLS (405) (Rvs) *Bam*HI*	GCAGCAGGATCCGAGCTTCTCACCTTTCTTGTGAATTCTTC	- sense
NLS (369) (Fwd) *Nco*I*	CATATGGCCATGGGCACAAAGAAACTAATGAAAATCACG	+sense

nents of the nuclear import machinery such as the monomeric guanine nucleotide binding protein Ran. A number of proteins have been shown recently (40–43) to be able to be imported into the nucleus through an analogous pathway involving direct interaction with importin β independent of importin α.

A functional NLS within residues 369–405 of Dengue NS5, predicted to be an interdomain region of NS5 linking the *S*-adenosylmethionine transferase and polymerase domains (44), has been identified and demonstrated to be functional. This region contains three basic clusters resembling NLSs that are separated by spacer regions of 14 and 11 amino acid residues and is recognized by importin *a/b* (44). More recently we used the yeast two-hybrid (Y2H) system to show that the adjacent residues 320–368 interact with importin β. Interestingly, we also showed that this same region also binds NS3 at either an identical or overlapping sequence (45).

In order to determine the functional significance of these sequences in the vicinity of the interdomain region of NS5, we set out to use site-directed mutagenesis and deletion analysis to define the essential elements of the importin *a/b*- and β-binding sites in NS5 nuclear localization for the first time. We show that nuclear targeting conferred by NS5-(369–405) is predominantly through residues 369–389 (the "a/bNLS"), which is recognized by importin *a/b*, and that the bNLS (residues 320–368) is able to direct β-gal to the nucleus of mammalian cells. Interestingly, the NS5-(369–405) region inhibits the ability of bNLS to transport β-gal to the nucleus and also prevents the binding of importin β to the bNLS, whereas the bNLS inhibits the importin *a/b*-binding and nuclear targeting abilities of the a/bNLS. This suggests that there may be important functional significance for the physical proximity of the a/b- and bNLSs in regulating the nuclear entry of NS5 during the late stage of Dengue infection, with the homology of these sequences comparable to gene products from a range of flaviviruses, implying wider importance.

MATERIALS AND METHODS

Cell Culture—Cells of the HTC rat hepatoma tissue culture line (a derivative of Morris hepatoma 7288C) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For *in vitro* nuclear transport assays cells were grown on glass coverslips for 2 days and then washed in intracellular buffer (110 mM KCl, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $CaCl_2$, 20 mM Hepes, pH 7.4, 1 μM dithiothreitol, 10 ng/ml leupeptin) just prior to mechanical perforation (46–50).

Plasmid Construction and Mutagenesis—Standard recombinant DNA techniques were used for plasmid construction and to introduce point mutations. Den2 NS5 NLS β-gal fusion constructs shown in Fig. 1 were constructed from plasmid pPR2-NS5-NLS (referred to as pPR2Nco-NS5CcN in Ref. 44), either after complete or partial removal of the DNA encoding NS5-NLS (corresponding to NS5 residues 369–405) as required, and insertion of DNA encoding the relevant new region of NS5 (residues 320–368). Prior to making the constructs shown in Fig. 1, plasmid pPR2-NS5-NLS was first subjected to partial *Bam*HI digestion followed by treatment with T4 DNA polymerase (Promega) in order to delete the *Bam*HI site downstream of the *lacZ* gene. The new plasmid pPR2-1B-NS5-NLS facilitated the cloning of relevant NS5 fragments into the unique *Bam*HI site at the 5′ end of *lacZ* to make the various fusion protein-expressing plasmid constructs used in this study.

Gene fragments were generated by standard PCR procedures using *Taq* polymerase (Promega), appropriate primers, and template. The NLS mutants and the a/bNLS were obtained by PCR utilizing pPR2-1B-NS5-NLS as a template and the combinations of the desired mutagenic primers, general primers, or primer for NS5-(386–405) (see Table I), except for the mutant KN123 which used a previously created mutant template pPR2-1B-NS5-NLSKN2 and the two mutagenic primers to give the remaining mutations. Plasmid pMJNS5 (45) was used as the template for amplification of regions for the cloning of the bNLS fusion constructs. Amplified products were subsequently digested with the appropriate restriction enzymes and ligated into pPR2-1B-NS5-NLS similarly digested with the corresponding restriction enzymes.

Plasmid pPR2-1B-NS5-a/bNLS (NS5 amino acids 369–391) was created by digestion of pPR2-1B-NS5-NLS with *Avr*II and *Bam*HI followed by digestion with mung bean nuclease and ligation. All plasmid constructs were verified by DNA sequencing. T-ag amino acids 111–135 fused to β-galactosidase (51) was used as a control.

Expression, Purification, and Fluorescent Labeling of β-Galactosidase Fusion Proteins—Escherichia coli strain MC1060 (52) bearing the plasmid encoding NS5-β-gal fusion protein was grown in LB medium supplemented with 100 μg/ml ampicillin and induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Purification of the fusion protein using an 4-aminophenyl-β-D-thiogalactopyranoside (Roche Molecular Biochemicals) affinity column and subsequent labeling with the fluorescent dye 5-iodoacetamidofluorescein was carried out essentially as described (46, 51).

In Vitro Nuclear Import Assay—In vitro nuclear import assays using mechanically perforated HTC cells in conjunction with confocal laser scanning microscopy (CLSM; Bio-Rad Radiance 2000) were performed as described previously (44, 46). Briefly, adherent HTC cells were grown on glass coverslips and mechanically perforated to generate intact nuclei that could be reconstituted to carry out nuclear import in the presence of exogenous cytoplasmic extract (untreated reticulocyte lysate; Promega), an ATP-regenerating system (0.125 mg/ml creatine kinase, 30 mM creatine phosphate, 2 mM ATP), and transport substrate (0.2 mg/ml of 5-(iodoacetamido)fluorescein-labeled fusion protein).

Image analysis for quantitation of fluorescence from CLSM was performed using NIH/Scion Image processing software as described previously (30, 48, 53). Curves were fitted for the function $Fn/c(t) = Fn/c_{max}(1 - e^{-kt})$, where t is time in minutes; Fn/c_{max} is the maximal

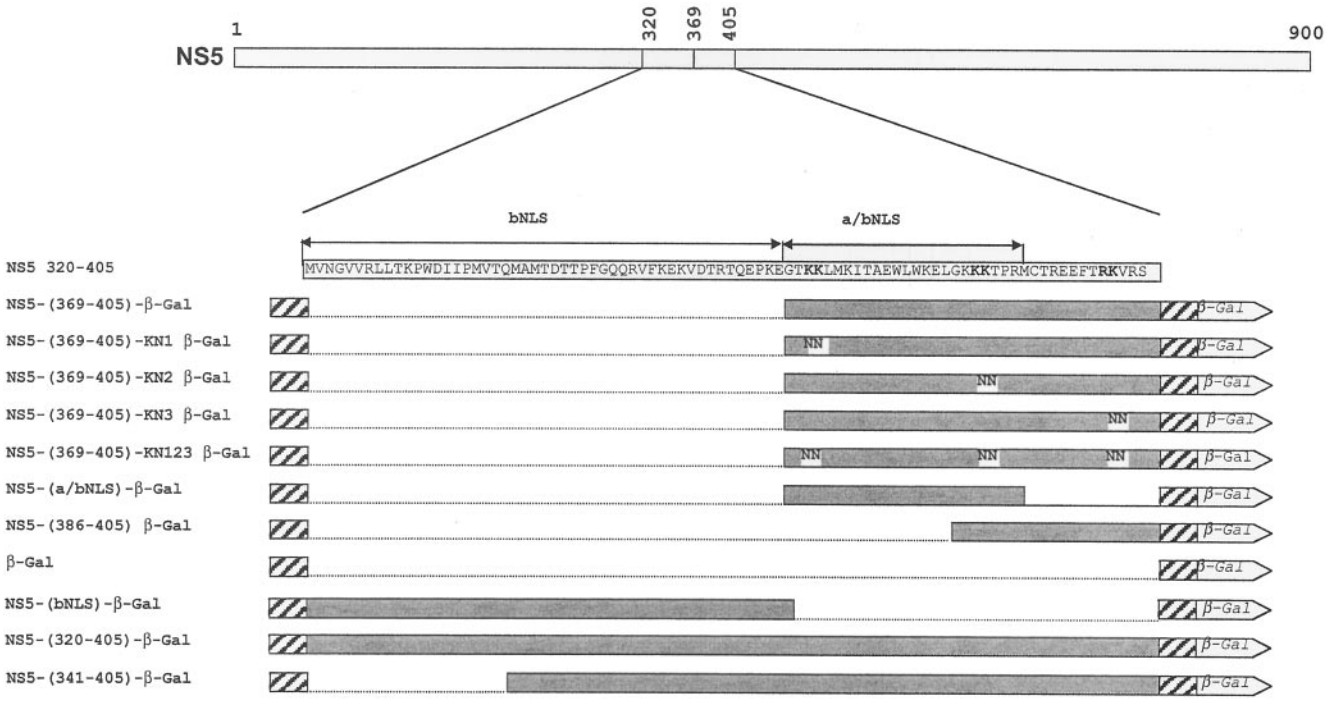

FIG. 1. **Schematic representation of NS5 and the NS5β-gal fusion proteins used in this study.** The NS5 amino acid sequence from residue 320–405 is presented in the *single letter* amino acid code, with *hatched boxes* indicating the linker region of the plasmid vector pPR2-Nco and the *shaded boxes* indicating the NS5 sequences. The basic residues (K or R) in the a/bNLS region that have been mutated to asparagine (N) are indicated in *bold* at the analogous positions in the mutant constructs. *Broken lines* indicate regions not included in the various fusion constructs. The importin β binding region (residues 320–368) is referred to as the bNLS in the text, by way of contrast to the a/bNLS region, which is recognized by the importin α/β heterodimer (see "Results").

level of nuclear accumulation; and k is the first-order rate constant (46, 54–56).

Importin Binding Assay—Mouse importin α– and importin β–glutathione *S*-transferase (GST) fusion proteins were expressed in *E. coli* and purified as described previously (48). The GST was cleaved from GST-importin α using thrombin, which was separated from the latter as described previously (57). Binding of importin subunits to β-galactosidase fusion proteins was determined using an ELISA-based binding assay as described (48, 56). Briefly, fusion proteins (0.5 μg/well) were coated overnight at 4 °C in 96-well microtiter plates (Nunc). After blocking, appropriate dilutions of GST-importin α, GST-importin β, or precomplexed GST-importin α/β were then added to the wells and incubated for 16 h at 4 °C. Bound importin was detected using goat anti-GST and rabbit anti-goat IgG alkaline phosphatase-conjugated antibodies (Sigma) and the colorimetric substrate *p*-nitrophenyl phosphate. A_{405} was followed with time using a plate reader (Molecular Devices), with values corrected by subtracting both the absorbance at 0 min and that for wells incubated without importins. Correction was made for differences in coating efficiency as described previously (48, 56) using a parallel β-galactosidase ELISA. Curves were fitted for the function $B(x) = B_{max}(1 - e^{-kx})$, where x is the concentration of importins, and k is the first-order rate constant (58).

GST Pull-down Assays of NS5 with GST-tagged Importin β or Its Complex with Importin α—Histidine-tagged NS5 and NS3 were expressed in *E. coli* strain AD494(DE3)(RIL) and purified using nickel-chelating chromatography.[2] 157.5 pmol of GST-importin β (157.5 pmol) or importin α/GST-importin β was added to 40 μl of glutathione-Sepharose 4B resin (Amersham Biosciences AB) in pull-down buffer (20 mM Tris-HCl, pH 7.9, 140 mM NaCl, 20 mM $MgCl_2$, and 0.1% Triton X-100) to a final volume of 100 μl and incubated at 4 °C with slow head-to-head mixing using a orbital rotor. The samples were centrifuged for 2 min at 7500 rpm at 4 °C, and the resin washed with 200 μl of pull-down buffer. The His-tagged NS5 (40.4 pmol) on its own or with equimolar His-tagged NS3 (44 pmol for 1 time and 440 pmol for 10 times) was added to the appropriate protein-bound resin in a final volume of 200 μl and incubated overnight (14–18 h) at 4 °C. The beads were washed 5 times with the pull-down buffer, and proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 60 mM reduced glutathione and 100 mM NaCl. The eluted fractions were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, detected with mouse polyclonal NS5 antibody (45), and visualized with ECL (Amersham Biosciences AB).

Homology Modeling—The crystal structure of the complex of importin β with the importin β binding (IBB) region of importin α (Protein Data Bank code 1QGK) was used to mutate the amino acid region 13–20 of importin α to the corresponding amino acids 353–360 of Den2 NS5 (see Fig. 6), and the packing of all mutated residues was assessed using the crystallography software package O (59). The potential collision between the side-chains of F17E and importin β Trp-342 was manually adjusted, and the model was refined by molecular dynamic energy minimization using crystallography NMR software (60). The root mean square deviations Cα atoms between the new model and the crystal structure is only 0.84 6Å, although the Cα of residues 15–18 moved around 1.1 to 1.7 Å. A new hydrogen bond (3.1 Å) was formed between E17 OE1 and Val-350 N.

RESULTS

The N-terminal Charge Cluster in the 37-Residue Interdomain Region of NS5 Is Critical for Nuclear Localization—We have previously localized a functional importin *a/b*-recognized NLS to the 37-amino acid interdomain region (residues 369–405) of Dengue 2 NS5 (44) and more recently shown that the N-terminally adjacent residues (320–368) interact with both importin β and the viral helicase NS3 (45). Residues 369–405 include three clusters of basic amino acid residues that resemble either three monopartite or two bipartite NLSs where the middle cluster of three positively charged lysine residues may be shared with either the N-terminal or C-terminal cluster. To test which of these residues are critical for NLS function, the lysine residues at positions 371 and 372 (KN1), 388 and 389 (KN2), and arginine at position 401 and lysine 402 (KN3) were altered by site-directed mutagenesis to asparagine separately or in combination (KN123, where all six positions were changed) as indicated (see Fig. 1). The encoded NS5-NLS-β-gal fusion proteins were overexpressed, purified, and fluorescently

[2] M. Johansson, A. J. Brooks, and S. G. Vasudevan, manuscript in preparation.

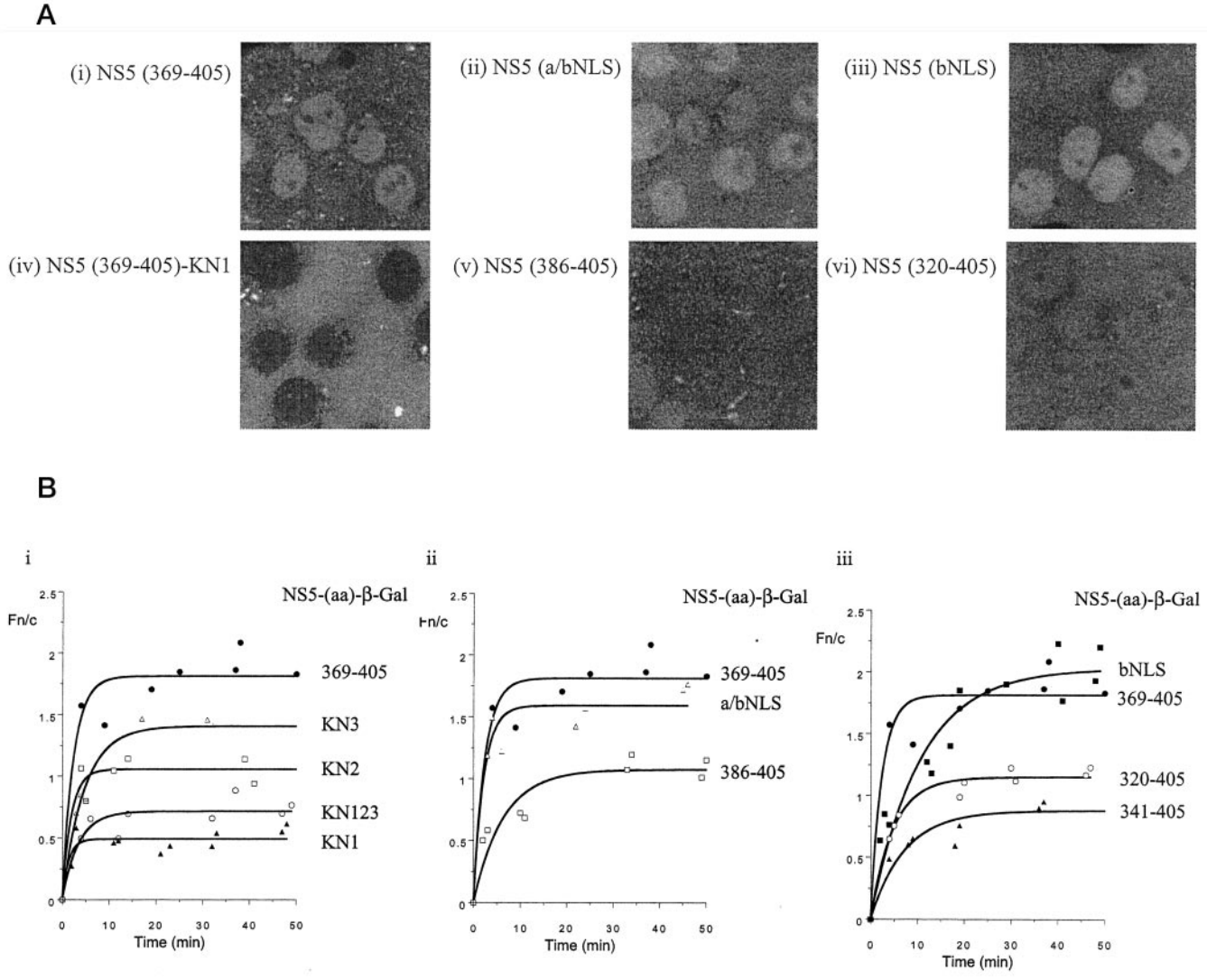

FIG. 2. ***In vitro*** **nuclear import of various constructs of the NS5 interdomain region fused to β-galactosidase.** *A*, CLSM images of nuclear accumulation after 35–40 min within mechanically perforated HTC cells in the presence of exogenous cytosol and an ATP-regenerating system of β-galactosidase fusion proteins containing NS5 residues (*i*) 369–405, (*ii*) 369–389 (also referred to as a/bNLS), (*iii*) 320–369 (also referred to as bNLS), (*iv*) NS5-(369–405)-KN1, (*v*) 386–405, and (*vi*) 320–405. *B*, quantitative data for nuclear import kinetics of NS5 interdomain β-galactosidase fusion proteins in mechanically perforated HTC cells. Experiments were performed as described in *A*. Results are for (*i*) NS5 residues 369–405 and the same residues carrying mutations in the three basic clusters KN1, KN2, and KN3 (see Fig 1); (*ii*) the two NLS truncations a/bNLS and NS5-(368–405) compared with NS5-(368–405); and (*iii*) bNLS compared with NS5-(369–405), NS5-(320–405), and NS5-(341–405). Data points represent the average of 6–8 separate measurements for each nuclear (Fn), cytoplasmic (Fc), and background (autofluorescence) fluorescence. Curves were fitted for the function $Fn/c(t) = Fn/c_{max}(1 - e^{-kt})$, where t is time in minutes; Fn/c is defined as the ratio of nuclear to cytoplasmic fluorescence; and k is the first-order rate constant (46, 56); regressions for the curve fits were not less than 0.92 and were in most cases >0.95. Results represent the average of three separate experiments with pooled data in Table II.

labeled (see "Materials and Methods") and then added to mechanically perforated HTC cells together with an ATP-regenerating system and exogenous cytosol to assess nuclear import kinetics using CLSM. Fig. 2 shows that NS5 amino acids 369–405 targeted β-gal to the nucleus maximally to levels about 2-fold those in the cytoplasm (Fn/c_{max} = 1.9) (44). β-Gal itself was excluded from the nucleus (Fn/c_{max} of 0.5, see Table II). Examination of KN1, KN2, KN3, and KN123 (Fig. 2*B*, *i*) indicated that both the Fn/c_{max} and $t_{½}$ (the time at which half-maximal nuclear accumulation is achieved) values were altered (Table II). The mutations in KN3 had the least effect compared with wild-type, whereas the nuclear import data for KN2 indicated an Fn/c_{max} of around 1.0. More drastic reductions in Fn/c_{max} were evident for KN1 and KN123, where the level of nuclear accumulation was not significantly above that of β-gal alone (Table II). Taken together, this suggests that the charged clusters KN1 and KN2 represent critical elements of the NLS.

To confirm this, plasmid constructs expressing β-gal fusion proteins carrying either the N-terminal (amino acid residues 369–391) or C-terminal (amino acid residues 386–405) portion of the NS5 interdomain region were derived (see Fig. 1), and the proteins were expressed, labeled, and assayed for nuclear import. Fig. 2, *A* and *B*, *ii*, show that residues 369–389 confer nuclear accumulation to a level comparable with that conferred by residues 369–405 (Table II). In contrast, residues 386–405 conferred poor nuclear accumulation (Fn/c_{max} of around 1). The results with the truncated constructs indicated that residues 369–389 constitute the minimal NLS of the NS5 interdomain and were thus consistent with the results for site-directed mutagenesis where the mutations of residues 371 and 372 affected the greatest reduction in nuclear accumulation of β-gal. We denote residues 369–389 the a/bNLS, recognized by

importin α/β (see below), to differentiate it from the bNLS (renders 320–368).

The NS5 Importin β Binding (bNLS) Region of NS5 Is a Functional Nuclear Localization Signal—We next addressed the question of whether the importin β binding region (bNLS, residues 320–368) of NS5 (45), immediately N-terminal to the a/bNLS, is able to function as an NLS. Fusion protein NS5-bNLS-β-gal (Fig. 1) was expressed, purified, fluorescently labeled, and examined in the *in vitro* transport assay using mechanically perforated HTC cells. Fig. 2*B*, *iii*, shows that although NS5-bNLS-β-gal accumulated in the nucleus of the HTC cells marginally better than NS5-NLS-(369–405)-β-gal, accumulation of the former was significantly ($p < 0.006$) slower, with the $t_{½}$ of ~10 min over 3-fold higher than that of fusion proteins containing NS5-(369–405) or the a/bNLS ($t_{½}$ of ~2–3 min) (Table II). Clearly, however, the bNLS is a functional NLS.

TABLE II
In vitro nuclear import kinetics of NS5 peptide-β-galactosidase fusion proteins

From experimental data such as that presented in Fig. 2*B*, curves were fitted for the function $Fn/c(t) = Fn/c_{max}(1 - e^{-kt})$, where t is time in minutes; Fn/c_{max} is the maximal level of nuclear accumulation, and k is the first-order rate constant (46, 56). Results shown are for mean ± S.E., with n indicated. $t_{½}$ is the time taken to achieve half-maximal level of nuclear accumulation.

NS5-β-Gal fusion	Fn/c_{max}	$t_{½}$
NS5-(369–405)-β-Gal	1.90 ± 0.26	2.22 ± 0.56
NS5-(369–405)-KN1-β-Gal	0.60 ± 0.14	ND[a]
NS5-(369–405)-KN2-β-Gal	1.09 ± 0.23	5.40 ± 0.34
NS5-(369–405)-KN3-β-Gal	1.48 ± 0.06	4.94 ± 0.27
NS5-(369–405)-KN123-β-Gal	0.75 ± 0.07	2.78 ± 1.15
NS5-(a/bNLS)-β-Gal	1.68 ± 0.25	2.67 ± 0.87
NS5-(386–405)-β-Gal	1.13 ± 0.33	ND
β-Gal	0.48	ND
NS5-(320–405)-β-Gal	1.13 ± 0.07	3.79 ± 1.28
NS5-(bNLS)-β-Gal	2.00 ± 0.30	9.70 ± 1.62
NS5-(341–405)-β-Gal	0.92 ± 0.27	7.02 ± 0.86

[a] ND, not able to be determined due to low import.

Mapping studies using the Y2H system and full-length and subdomain constructs of NS5 had indicated that the bNLS region (residues 320–368) is important for competitive binding of importin β and NS3. Surprisingly, constructs that contained both the bNLS and the 37-amino acid NLS region (residues 320–405) displayed only very weak interaction with importin β in the Y2H assay (45). To explore this phenomenon of inhibition of importin β binding by NS5 residues 369–405, a β-gal fusion protein containing NS5 residues 320–405 was tested for its ability to accumulate in the nucleus of HTC cells. NS5-(320–405)-β-gal construct showed markedly reduced accumulation compared with both the bNLS- or NS5-(369–405)-containing β-gal fusion proteins (Fig. 2*B*, *iii*; Table II). Fusion protein NS5-(341–405)-β-gal was also poorly transported to the nucleus (Fig. 2*B*, *iii*; Table II), indicating that residues 320–340 were not specifically responsible for this effect.

Importin β or α/β Can Bind Directly to the NS5 bNLS/NLS Region—Although the bNLS clearly represented a functional NLS additional to the previously characterized a/bNLS, the

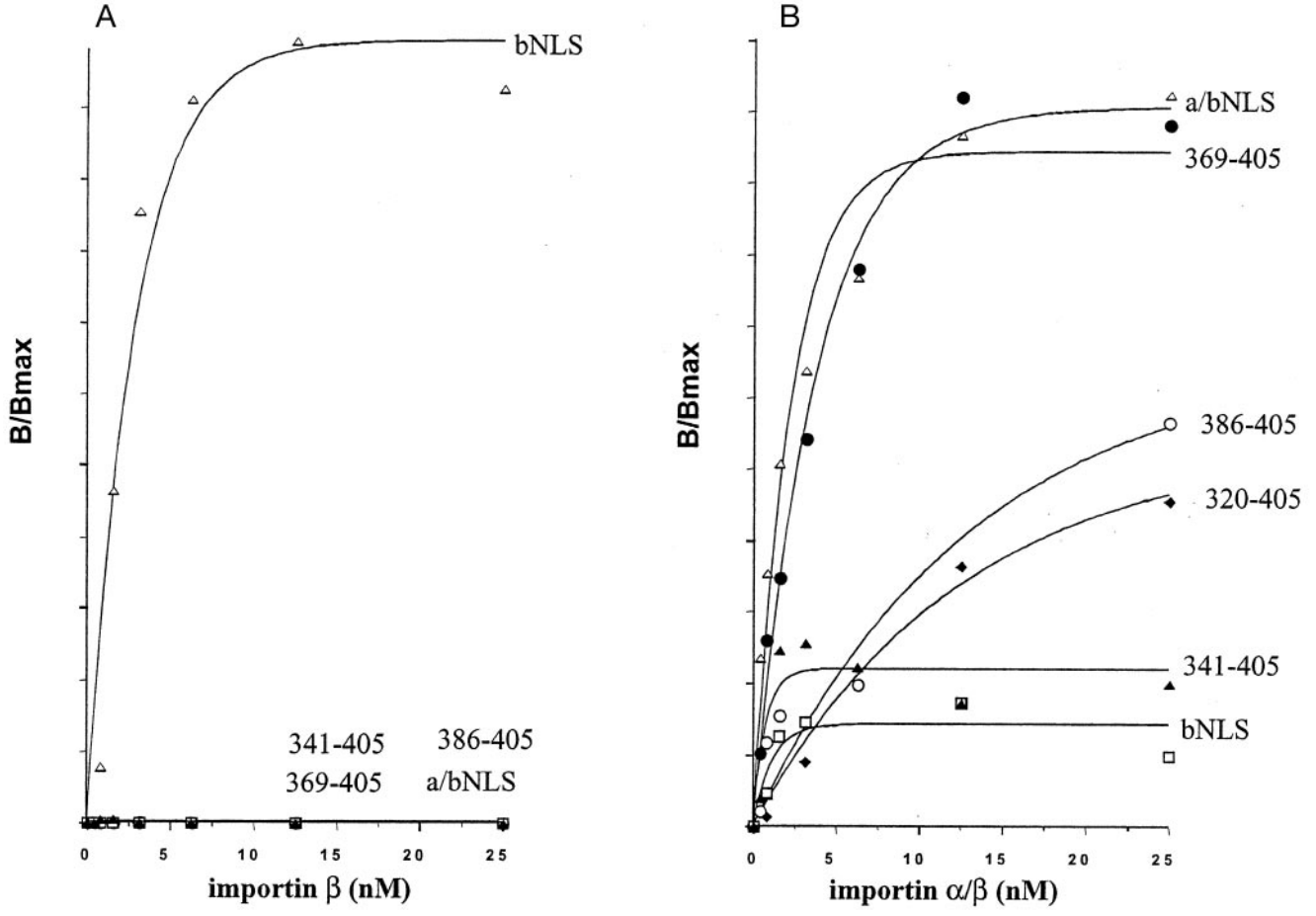

FIG. 3. **Measurement of the affinity of binding of importins to NS5-β-galactosidase fusion proteins as determined using an ELISA-based binding assay.** Measurements were performed as described under "Materials and Methods" (48, 56) and fitted to the function $B(x) = B_{max} \cdot (1 - e^{-kt})$, where x is the importin concentration. The results are from a single typical experiment performed in triplicate, with pooled results in Table III.

TABLE III
Affinities of importin binding to NS5-β-gal fusion proteins as determined using an ELISA-based binding assay

NS5-β-Gal Fusion	Importin binding affinity (nM)[a]		
	Importin α	Importin β	Importin α/β
NS5-(369–405)-β-Gal	430 ± 180 (2)	ND[b] (2)	27.3 ± 6.6 (3)
NS5-(a/bNLS)-β-Gal	616 ± 195 (1)	ND (1)	21.7 ± 3.0 (3)
NS5-(386–405)-β-Gal	760 ± 225 (1)	ND (1)	121 ± 21 (3)
NS5-(320–405)-β-Gal	1340 ± 405 (1)	ND (1)	139 ± 16 (3)
NS5-(bNLS)-β-Gal	ND (2)	23.5 ± 4.5 (2)	ND (2)
T-ag-NLS-β-Gal	13 ± 2 (2)	ND (1)	3.1 ± 0.4 (2)

[a] Importin binding was measured as described under "Materials and Methods" (48, 56), and the experimental data (see Fig. 3) were fitted for the function $B(x) = B_{max} * (1 - e^{-kt})$ where x is the concentration of importin α, importin β, or the importin α/β heterodimer. Results for the apparent dissociation constant (K_d) represent the mean ± S.E. (n in parentheses); where $n = 1$; S.E. was obtained from curve fit. Results for T-ag-NLS-β-gal, carrying T-ag amino acids 111–135, including the NLS, are shown by way of comparison.
[b] ND, not able to be determined due to low binding.

results from the *in vitro* nuclear transport assay did not enable us to exclude the possibility that the observed nuclear accumulation was due to the action of importin α/β because both importin α and β are present in the exogenous cytosol added. To clarify this, direct binding of importin α, importin β, or the importin α/β heterodimer to the various fusion proteins expressing the different NS5 regions were assessed using an ELISA-based assay (see "Materials and Methods").

Fig. 3*B* (see also Table III) clearly shows that only NS5-bNLS-β-gal was recognized strongly by importin β with a K_d in the low nanomolar range. None of the other fusion proteins tested, including the construct that contained both the bNLS and NLS (*i.e.* residues 320–405), displayed any measurable recognition by importin β in the absence of importin α. Clearly, the bNLS region of NS5 is recognized by importin β, but the additional presence of residues 369–405, including the a/bNLS, prevents importin β from binding to that site, implying a masking and/or conformational effect.

Binding studies with importin α alone and the importin α/β heterodimer confirmed the strong binding previously observed with NS5-NLS-β-gal (44); as observed previously, binding affinity was much greater for the latter because of the fact that in the absence of importin β importin α is autoinhibited (61, 62). The a/bNLS (residues 369–389) fusion protein construct was recognized by importin α/β with affinity comparable with NS5-(369–405)-β-gal, suggesting that residues 369–389 constitute the minimal importin α/β heterodimer binding region, and correlating nicely with the nuclear transport results (above) indicating that these residues represent the minimal region with full nuclear targeting function. The poorest binding for the importin α/β heterodimer was recorded for the bNLS construct fused to β-gal, whereas the NS5-(386–405) and the NS5-(320–405) constructs displayed weak but measurable binding with K_d values around 100 nM (Fig. 3; Table III).

Intriguingly, as for the bNLS where the presence of the a/bNLS impaired importin β binding, the NS5-(320–405) construct was not recognized with high affinity by the importin α/β heterodimer, indicating that the presence of the bNLS impinged on the recognition of the a/bNLS by importin α/β. Again, this was consistent with masking and/or conformational effects modulating importin access to the two NLSs.

Thus, NS5-(369–389), the a/bNLS recognized by the importin α/β heterodimer, and NS5-(320–369), the bNLS recognized by importin β, represent functional NLSs, recognized with high affinity by their respective nuclear import receptors. That proteins containing both NLSs are not recognized with high affinity by importins implies that there may be complex regulation of the importin binding process; because NS3 also binds to the bNLS region (45), it seems probable that competition for binding to NS5 between importins and NS3 occurs, which is likely to be of physiological significance in the context of Dengue infection.

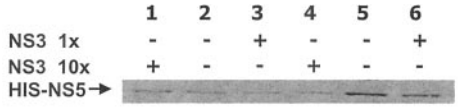

FIG. 4. **NS5 pull-down assay with importins.** Precomplexed importin α/GST-importin β (*lanes 1–3*) or GST-importin β (*lanes 4–6*) bound to Glutathione-Sepharose 4B resin were incubated with NS5 in the absence or presence of equimolar or excess (10 times) NS3 as indicated and subjected to a pull-down assay as described under "Materials and Methods." The pulled down fractions were separated by 10% SDS-PAGE and visualized by immunoblotting using a specific anti-NS5 polyclonal antibody.

NS3 Binds the bNLS Site but Not the a/bNLS—We had shown previously that GST-importin β can pull-down full-length His-tagged NS5, confirming the interaction between these two proteins originally observed using the Y2H system (45), whereas binding of the importin α/β heterodimer to NS5-NLS-β-gal has been shown previously as well as in the present study (44). In order to demonstrate that purified full-length NS5 can also interact with the importin α/β heterodimer and to investigate the level to which the NS3-binding site overlaps with the bNLS and a/bNLS sites, pull-down assays were carried out as described under "Materials and Methods." The ability of either importin α/β heterodimer or importin β to pull-down NS5 either in the absence (Fig. 4, *lanes 2* and *5,* respectively) or presence of equimolar (*lanes 3* and *6*) or 10-fold excess (*lanes 1* and *3*) of NS3 was analyzed by SDS-PAGE separation followed by Western blotting (Fig. 4). The result clearly shows for the first time that, through the a/bNLS, the importin α/β heterodimer interacts with full-length NS5. Based on the amount of the NS5 pulled down (compare *lanes 2* and *4*), it is possible to conclude that the binding affinity of the importin α/β heterodimer for NS5 may be lower than that of importin β. This is despite the similar K_d value observed for the binding of the NS5 peptides fused with β-gal in the ELISA-based binding assay (see Table III), but this is not entirely surprising because the peptide binding may not be subject to the same steric constraints as the full-length protein. As shown previously, an unrelated coral protein fused to GST (45) and GST importin α did not pull-down NS5 (data not shown). Also, the amount of NS5 pulled down when importin α was added at the same time as NS5 to GST-importin β was similar to that observed with the precomplexed importin heterodimer (data not shown).

Arguably the most interesting result from Fig. 4 is that NS3 competes for the bNLS site that is recognized by importin β, as described previously (45), whereas binding of the importin heterodimer α/β to the a/bNLS site is unaffected by even a 10-fold molar excess of NS3 as indicated by the similar amount of NS5 pulled down (Fig. 4, *lanes 1–3*). This implies that while NS3 can compete for the bNLS with importin β, it is not able to compete for the a/bNLS with importin α/β.

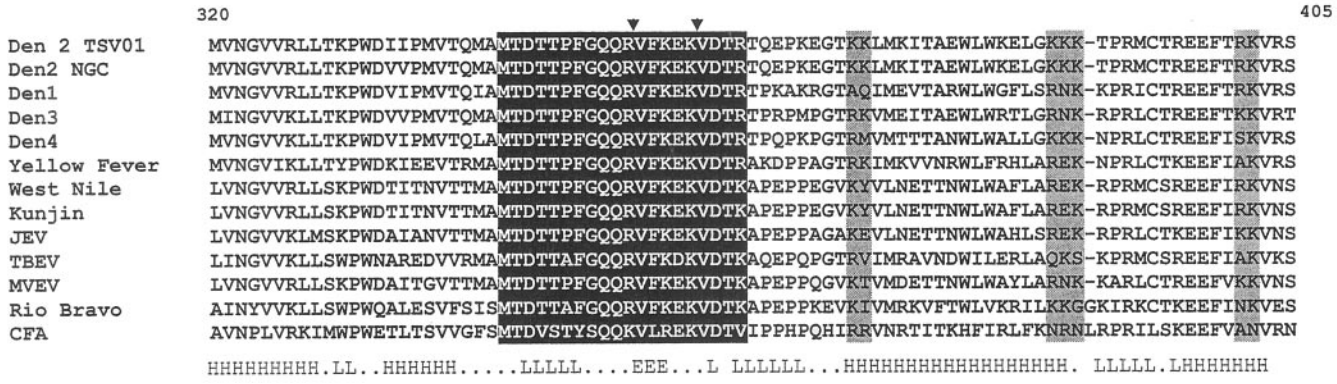

FIG. 5. **Alignment of sequences in the NS5-(320–405) region of Dengue 2 virus with other Dengue subtypes and representative members of the flavivirus genus.** The three basic clusters KN1, KN2, and KN3 are *shaded* in *light gray*. The highly conserved 20-amino acid region within the bNLS is highlighted by a *black background*. A *dash* is used to signify a gap to give optimal alignment. The virus sequences shown (and their GenBank™ accession numbers) are as follows: Den2 TSV01 (AY037116), Den2 NGC (AF038403), Den1 (U88535), Den3 (M93130), Den4 (AF32657), Yellow Fever (X15062), West Nile (M12294), Kunjin (D00246), Japanese encephalitis virus (M55506), tick-borne encephalitis virus (U27495), Murray Valley encephalitis virus (AF161266), Rio Bravo (AF144692), cell-fusing agent (M9167). The residues Arg-353 and Lys-358 that are significant for binding to importin α (see Fig. 6) are indicated by the *arrowheads*. Shown *below* the alignment is the secondary structure prediction using the PHD program of Rost and Sander (66): *H*, helix; *L*, loop; *E*, strand; *dot*, not able to be predicted.

DISCUSSION

We have shown previously (44, 45) that the interactions of NS5 with the virus-encoded NS3 protein and other cellular factors such as importin α or importin α/β heterodimers map to a small sequence region within NS5 spanning residues 320–405. Within this region, the 37-amino acid linker sequence ($^{369}X_2$KKX_{14}KKKX_{11}RK$X_3{}^{405}$) contains the signal that can target the carrier β-gal (476 kDa) to the nucleus (44). In this study we pinpoint lysine residues at positions 371 and 372 of Den2 NS5 as critical components of the signal for nuclear targeting. By using site-directed mutagenesis we altered these two residues to asparagine (hydrophilic and neutral side-chain) in the NS5 linker fused to β-gal, and we demonstrated that the ability of the mutant NS5 sequence to transport β-gal to the nucleus was abolished, *i.e.* the mutant fusion protein NS5-KN1-β-gal is excluded from the nucleus in comparable fashion to β-gal itself (Fig. 2, *iv*). Similar analysis of the central and C-terminal clusters of basic residues (KN2 and KN3) within the linker region indicates that NS5 residues 369–389 contain the NLS. Two observations support this assignment as follows: the demonstration of a similar level of nuclear localization of β-gal fused to either NS5 residues 369–389 (a/b NLS) or residues 369–405 in the *in vitro* nuclear transport assay, and the binding affinity of the importin α/β heterodimer is comparable for both NS5-a/bNLS-β-gal and NS5-(369–405)-β-gal.

We also show for the first time that the importin β-recognized NS5 region 320–368 (bNLS), which was identified using the Y2H system, is a functional NLS. Intriguingly β-gal fusion proteins that contain both bNLS and a/bNLS do not accumulate in the nucleus, suggesting that these two NLSs are engaged in some inhibitory interaction that prevents either signal from facilitating nuclear transport. This counteraction by the two NLSs has been shown using three independent methods. First, the Y2H results indicate almost no interaction between NS5 residues 320–405 and importin β or NS3 (45) but strong interactions of both with NS5 residues 320–368. Second, the results from the ELISA-based binding assay shown here show that importin β binds to NS5-bNLS-β-gal with nanomolar affinity but does not bind NS5-(320–405)-β-gal; similarly, importin α binds to residues 369–405 but not in the presence of residues 320–368 or 341–368. Finally, the nuclear transport of NS5-(320–405)-β-gal is greatly reduced by comparison with NS5-(320–368)-β-gal and NS5-(369–405)-β-gal, indicating that either the a/bNLS or bNLS is poorly functional in the presence of the other NLS. The mechanism of this apparent inhibitory interaction is difficult to ascertain at present, but it is tempting to speculate that it may indeed be an example of an intrasteric autoinhibition regulatory mechanism (62) centered around what appears to be a "hotspot" for protein interaction in NS5 (see below).

Our approach to understanding the interaction between Dengue NS5 and NS3 proteins has focused on the interdomain region of Dengue 2 NS5 that contains the functional bNLS and a/bNLS. Significantly, NS3 competes with importin β for the bNLS site (45). The importance of the NS5 interdomain region (residues 320–405) is also supported by alanine mutagenesis of different charged residues in a full-length infectious clone of Dengue 4, which demonstrated that the NLS region is critical for virus viability (63). In particular, mutation of residues 356/357 (likely to be important for interaction with importin β, see Figs. 5 and 6 below), 387/388 (the equivalent of our KN2 mutant), or 388/389 (within the a/bNLS) all impairs viral replication (63), consistent with their role in interacting with importins and of course NS3 being of physiological importance. However, the primary sequence alignment of the NS5-(320–405) region of Dengue subtypes and other members of the flavivirus genus reveal strong sequence conservation within residues 386–405 which indicates a functional role. The sequence alignment also reveals that the bipartite a/bNLS is not readily apparent in Dengue 1 NS5 but is noticeable in Dengue 2–4 by comparison with other bipartite NLSs (35). In Dengue 2 NS5, the KN1 charge cluster (Lys-371 and Lys-372) is critical for nuclear localization as shown here, and similar dibasic residues are also present at the homologous site in YFV NS5 that has been shown to localize in the nucleus (28). The absence of basic residues at positions 371/372 in Dengue 1 NS5 raises the question whether the Dengue serotypes that have not been experimentally demonstrated to localize in the nucleus will indeed do so, because the bipartite NLS appears to be absent. Interestingly a candidate T-ag-type monopartite NLS (^{363}PKAKRG) (see Introduction) is present in Dengue 1 interdomain linker region which suggests that yet further nuclear localization mechanisms may operate.

The most important result in the sequence alignment (Fig. 5) is the almost complete conservation of a 20-amino acid sequence (^{342}AMTDTTPFGQQRVFKEKVDT) within the bNLS. The cell fusing agent virus that is tentatively classified as a member of the flavivirus genus (64) showed the greatest deviation from this conserved sequence motif. The significance of this region was demonstrated in a previous study by Khromykh

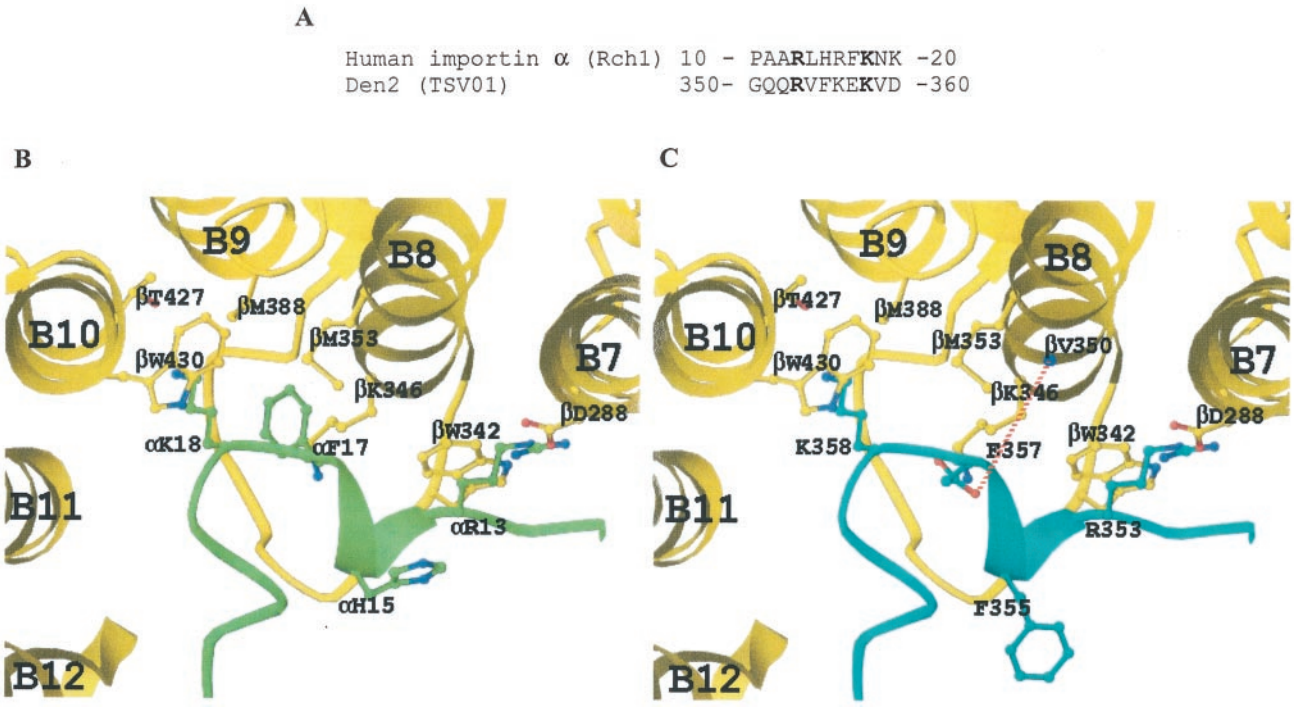

FIG. 6. **Molecular model of importin β interaction with the NS5 bNLS region.** *A*, primary sequence comparison of the N-terminal portion of the importin *α* IBB domain (human-hSRP1*α*; Rch1) and NS5 bNLS residues 350–360. *B*, interactions of the N-terminal portion of the importin *α* IBB domain (*green*) with the C-terminal helix of importin *β* (*yellow*) (69). *C,* model of interaction of the bNLS region residues 350–360 with importin *β*, generated as described under "Materials and Methods" using the GRASP and RIBBONS programs, based on the Protein Data Bank coordinates for the importin *α* peptide from Cingolani *et al.* (69) (see also "Discussion").

and colleagues (65) using *trans*-complementation studies with a helper system that stably expressed the non-structural proteins of Kunjin virus. They showed that the deletion of the RNA encoding the entire polymerase domain of NS5 could be complemented, but the additional removal of the RNA encoding the 20-amino acid sequence within bNLS severely reduced rescue of the defective virus. The conserved 20-amino acid region has been referred to as "motif c" of the N-terminal region of NS5 and was speculated to bind NS3 and other non-structural proteins such as NS2A (65). Our previous demonstration that the helicase domain of Den2 NS3 binds to this conserved region of NS5 by Y2H assay and also biochemically in a pull-down assay (45) confirmed the speculation.

Secondary structure prediction of the conserved 20-amino acid sequence suggests that it most probably forms a loop region (66). Consistent with this is the idea that loops in protein ligands are the preferred motifs for promiscuous binding to different protein targets as is the case with signal transduction proteins that interact with multiple targets (67, 68). Structural studies have shown that part of the molecular recognition between importin *β* and importin *α* occurs when the N-terminal part of the IBB (importin *β* binding) domain of the latter uses its two invariant residues, *α*Arg-13 and *α*Lys-18 (conserved in all importin *α*s), to snuggle into a spatially conserved arrangement of acidic and hydrophobic residues in importin *β* (Fig. 6) (69). Intriguingly, within the conserved 20-amino acid region of NS5 there are two invariable residues Arg-353 and Lys-358 that are identical and similarly spaced to the conserved residues in importin *α* IBB. It is also very interesting that the *α*17 position of the IBB which is either Phe or Tyr (in all importin *α*s) may be replaced by Glu (present at the analogous site of NS5) because its carboxylate group is within H-bonding distance from *β*Lys-346, but homology modeling suggests that H-bonding of the Glu-17 OE1 occurs with the main chain amide nitrogen of *β*Val-350 (Fig. 6). Furthermore, the predicted *β* strand within "motif c" (residues 354–356) forms a 3_{10}-helix similar to the importin *α* IBB sequence and consequently the bulky aromatic side chain of NS5 Phe-356 points away from the binding pockets for Arg-353 and Lys-358. Our homology model of the NS5 motif c region is in agreement with the demonstration here that importin *β* binds strongly to the bNLS region.

Whether the importin *β* binding that has been mapped to this conserved region and shown here to be functional in nuclear transport assays is relevant for virus morphogenesis and/or pathogenesis is not yet clear, but based on all the available evidence this region of NS5 certainly warrants detailed study. We have already drawn attention to the fact that NS5A from hepatitis C virus, a member of the *Flaviviridae* family, interacts with an importin *β* homologue known as karyopherin-*β*3 (45). In that case, a putative hijacking role was ascribed for hepatitis C virus NS5A which suggested that the viral protein may sequester nuclear transport receptors and interfere with transport of cellular proteins to the nucleus (70). Consistent with the significantly slower rate of bNLS-mediated nuclear transport, relative to that mediated by the a/bNLS (see Table II), an interesting possibility in this context is that the bNLS may act through importin *β* as some sort of cytoplasmic retention signal for NS5, sequestering importin *β* at the same time, and preventing it from fulfilling its normal transport role.

Based on our results here and previously (45) with respect to the protein interactions we have identified using Y2H and *in vitro* techniques, we propose that a concerted series of events are likely to occur in a Dengue virus-infected mammalian cell. First, after synthesis the NS5-(320–405) region is most likely masked because of its interaction with NS3 (45, 65). Subsequent autoproteolysis of NS3 (71, 72) may expose the NS5-(320–368) region and allow importin *β* to bind the NS5 bNLS (the binding affinity of this interaction is comparable with that of importin *β* for importin *α*). Importin *α* is then recruited to this site and as a result of a conformational change the impor-

tin α/β heterodimer then binds to the a/bNLS region and subsequently transports NS5 to the nucleus with reasonably high efficiency to impact on host cell nuclear functions. Lower efficiency importin β-mediated nuclear transport may account for the cytoplasmic retention phenomenon that we have observed previously (44), but where phosphorylation may play a role in all of this is unclear. Of course, hard experimental evidence is needed to address the details of the various protein-protein interactions within the NS5-(320–405) region using full-length viral and host proteins. Based on our analysis, carefully selected mutations in this region may well assist in the development of an effective and rationally designed live attenuated virus, making a Dengue vaccine a step closer to being a realistic possibility. Finally, the high level of conservation of the bNLS region (Fig. 5) makes it an attractive potential target for a broad-acting bifunctional antiviral agent against flaviviruses that utilize the same replicative mechanism as Dengue.

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The Interdomain Region of Dengue NS5 Protein That Binds to the Viral Helicase NS3 Contains Independently Functional Importin β1 and Importin α/β-Recognized Nuclear Localization Signals

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