ELSEVIER

Biochimica et Biophysica Acta 1538 (2001) 260–272

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

LIM domain protein Trip6 has a conserved nuclear export signal, nuclear targeting sequences, and multiple transactivation domains

Yuan Wang, Thomas D. Gilmore *

Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215-2406, USA

Received 26 September 2000; received in revised form 4 January 2001; accepted 18 January 2001

Abstract

Trip6 is a member of a subfamily of LIM domain proteins, including also zyxin, LPP, Ajuba, and Hic-5, which localize primarily to focal adhesion plaques. However, in this report, we demonstrate that Trip6 is largely in the nucleus in cells treated with leptomycin B, suggesting that Trip6 shuttles between nuclear and cytoplasmic compartments and that nuclear export of Trip6 is dependent on Crm1. Consistent with this finding, we have identified a nuclear export signal (NES) in Trip6, and mutation of this NES also results in sequestration of Trip6 in the nucleus. Addition of the Trip6 NES to the nuclear v-Rel oncoprotein redirects v-Rel to the cytoplasm. Trip6 also has at least two sequences that can direct cytoplasmic β-galactosidase to the nucleus. Using GAL4 fusion proteins and reporter gene assays, we demonstrate that Trip6 has multiple transactivation domains, including one that appears to overlap with sequences of the NES. In vitro- or in vivo-synthesized Trip6, however, does not bind to DNA–cellulose. Taken together, these results are consistent with Trip6, and other members of this LIM protein family, having a role in relaying signals between focal adhesion plaques and the nucleus.

Keywords: Trip6; Zyxin; LIM domain; Transcriptional activation; Leptomycin B; Nuclear export signal

1. Introduction

LIM domains are thought to be primarily protein–protein interaction domains [1,2]. Trip6 is a LIM domain-containing protein that is part of a subfamily of LIM domain proteins that also includes zyxin, lipoma preferred partner (LPP), Ajuba, paxillin, and Hic-5, among others [1,2]. These proteins contain three or more copies of highly related LIM domains towards their C-termini.

The exact functions of members of this subfamily of LIM domain proteins are not known. In resting cells, most proteins in this family primarily reside in focal adhesion plaques, but under a variety of circumstances several can also enter the nucleus [3–8]. Moreover, LPP and Trip6 have sequences that can activate transcription [5–7], and Hic-5 can bind DNA through its LIM domain sequences [3]. Zyxin and LPP have been shown to interact with several focal adhesion plaque proteins, including Ena/VASP [5,9,10]. In addition, Trip6 has been identified as interacting with a variety of nuclear (thyroid hormone receptor [11] and v-Rel [7]) and cytoplasmic (protein phosphatase PTP1E and PTPBL [12,13]) proteins. Taken together, these results suggest that these LIM proteins have distinct nuclear and adhesion plaque functions, and may relay signals between focal adhesion plaques and the nucleus.

* Corresponding author. Fax: +1-617-353-6340;
E-mail: gilmore@bu.edu

0167-4889/01/$ – see front matter
PII: S0167-4889(01)00077-5

In this report, we have further investigated the requirements for nuclear activities of Trip6. Our results demonstrate that Trip6 appears to shuttle between cytoplasmic and nuclear compartments, due to a nuclear export signal (NES) towards its N-terminus and nuclear targeting functions in both N-terminal sequences and in C-terminal sequences that include the LIM domains. We also show that Trip6 has multiple transactivation domains, including one that appears to overlap with the NES.

2. Materials and methods

2.1. Recombinant DNA techniques and plasmid constructions

Recombinant DNA techniques were generally performed as described [14]. Site-directed mutagenesis of the NES of Trip6 was performed by PCR using the following oligonucleotides as the mutagenic primers and pcDNA-FLAG-Trip6 [6] as template: 5′-CATCAGCCGCCATGCTGGTGGCCGAATCTATCTC-3′ (triple-mutation primer 1) or 5′-GATCAGCCGCCATGCTGGTGGCCGAATCTATCTC-3′ (double-mutation primer 1) or a Trip6-specific primer (for single mutation) and T7 promoter primer to generate 5′ NES/mutant PCR product; 5′-AGATTCGGCCACCAGCATGGCGGCTGATGCGGACGGGGGT-3′ (triple-mutation primer 2) or 5′-AGATTCGGCCACCAGCATGGCGGCTGATCTGGACG-3′ (double-mutation primer 2) or 5′-GATTCGCTCACCAGCATGTTGGCTGATGCGGACGGGGGTCGC-3′ (single-mutation primer 2) and a downstream Trip6-specific primer to generate 3′ NES/mutant PCR product. After digestion of the NES/mutant PCR products with *Eco*RI/*Bst*XI and the 3′ NES/mutant PCR products with *Bst*XI and *Sac*II, these two fragments were simultaneously subcloned into pcDNA-FLAG-Trip6, which had been digested with *Eco*RI and *Sac*II.

Retroviral vectors for the expression of v-Rel, v-dStu/Hinc, and TGV have been described previously [15]. To create plasmids v-Rel-NES and v-Rel-NES-mutant, primers were used to amplify the NES-containing region of wild-type and NES-mutant Trip6. The fragments were then digested with *Stu*I and *Hin*cII, which digested within the designed primers, and the fragments were used to replace a 120 bp fragment of v-*rel* between unique *Stu*I and *Hin*cII sites [15]. The recombinant v-*rel-trip6/NES* chimeric genes were then subcloned as *Xba*I fragments into the corresponding site of spleen necrosis virus vector JD214BS+ [16].

To create a plasmid for the expression of β-galactosidase (β-gal), the *lacZ* gene with 5′ sequences of the CAT gene was digested by *Hin*dIII and *Dra*I in plasmid RSV-β-gal [15], and this fragment was subcloned into *Hin*dIII/*Eco*RV-digested pcDNA3.1(+). To create plasmids expressing the β-gal-LIM and β-gal-ΔLIM fusion proteins, a C-terminal fragment or the N-terminal region was generated by *Sca*I/*Xba*I or *Hpa*I/*Xba*I digestion of pcDNA-FLAG-Trip6, respectively, and these fragments were then ligated to *lacZ* (in-frame) in pcDNA3.1-β-gal that had been treated with *Blp*I/Klenow and *Xba*I. To create pcDNA3.1-β-gal-ΔLIM/NES mutant, the *Kpn*I/*Bsp*EI fragment from pcDNA3-Trip6/NES mutant was subcloned into pcDNA3.1-β-gal treated by *Kpn*I/*Bsp*EI. All subclones were confirmed by DNA sequencing and in vitro translation.

The GAL4 producer plasmid SG424 and GAL4–Trip6 producer plasmid SG-MoTrip6 (GAL4-ΔN-Trip6-1) have been described previously [6,17]. pcDNA-FLAG-Trip6-LIM and pcDNA-FLAG-Trip6-ΔLIM were produced by *Eco*RI/Klenow and *Sca*I or *Not*I/Klenow and *Sca*I digestion of pcDNA-FLAG-Trip6, respectively, and the vector backbones were then religated. GAL4–Trip6, GAL4–Trip6-LIM and GAL4–Trip6-ΔLIM were then created by subcloning *Hpa*I and *Xba*I fragments from corresponding pcDNA-FLAG plasmids (described above) into pSG424 that had been treated with *Kpn*I/T4-DNA-polymerase and *Xba*I. GAL4-ΔN-Trip6-2 was made by subcloning an *Eco*RI/Klenow- and *Xba*I-treated fragment from pcDNA-FLAG-ΔN-Trip6 [6] into pSG424 that had been treated with *Bam*HI/Klenow and *Xba*I. To create pcDNA-FLAG-ΔN-Trip6-3, an *Nco*I/Klenow-treated to *Not*I fragment of a mouse Trip6 cDNA was subcloned into pcDNA-FLAG that had been digested with *Bam*HI, treated with Klenow and then digested with *Not*I. GAL4-ΔN-Trip6-3 was then made by subcloning a *Bam*HI/*Xba*I-digested fragment from pcDNA-FLAG-ΔN-Trip6-3 into the corresponding sites of pSG424. All pSG-Trip6/NES-mutants were

created by subcloning *Kpn*I/*Xba*I fragments from the given pcDNA-Trip6/NES mutants into pSG-Trip6 that had been digested with *Kpn*I and *Xba*I. Plasmid SG-zyxin was created by subcloning a fragment containing codons 1–572 of human zyxin into pSG424 that had been treated with *Kpn*I/Klenow/*Xba*I. All subclones that involved blunt-end ligations were confirmed by DNA sequencing.

2.2. Cell culture, GAL4-site reporter gene assays, and indirect immunofluorescence

Chicken embryo fibroblasts (CEF) were obtained from Spafas, Inc. and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Where indicated, leptomycin B (a kind gift of Minoru Yoshida) was added at 20 ng/ml. GAL4-site CAT reporter gene assays were performed as described previously [6]. Indirect immunofluorescence on transfected cells was performed by fixing cells in paraformaldehyde and using primary antiserum against FLAG, β-gal, or GAL4 as described previously [6,15,18]. Detection of antigens with fluorescein-conjugated secondary antibody and DAPI staining were also performed as described previously [6].

2.3. DNA-binding assays

Coupled in vitro transcription/translation was performed in wheat germ extract or rabbit reticulocyte lysate in the presence of Tran^{35}S-label (Amersham) according to the manufacturer's (Promega) recommendations as described previously [6]. FLAG epitope-tagged full-length Trip6 and N-terminally truncated Trip6(ΔN) were translated in vitro from pcDNA-FLAG-Trip6 and pcDNA-FLAG-ΔN-Trip6, respectively. Trip6, c-Src, v-Rel and v-Rel mutant (v-dStu/Hinc) were translated in vitro from pGEM4-MoTrip6, pGEM4-c-Src, CG129 and CG129/dStu/Hinc expression plasmids, respectively, which have been described previously [6,19]. Double-stranded DNA–cellulose (USB Corporation; 80 mg/ml) was preincubated in BD buffer (0.2 M NaCl, 50 mM Tris, pH 7.5, 0.2 mM $ZnCl_2$, 0.2% (v/v) Triton X-100, 5% (v/v) glycerol, 2 mg/ml BSA) at 4°C overnight, and 50 μl DNA–cellulose was then incubated with 5–10 μl of in vitro-translated proteins in BD buffer at room temperature for 2 h, washed once with BD buffer briefly, twice with wash buffer (0.3 M NaCl, 50 mM Tris, pH 7.5, 0.2 mM $ZnCl_2$, 1% Triton X-100, 0.1% SDS, 5% glycerol) for 10 min each at room temperature. DNA–cellulose beads were pelleted and boiled in 2×SDS sample buffer (0.125 M Tris, pH 6.8, 4.6% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.2% bromophenol blue) at 95–100°C for 20 min. The supernatant was then loaded on a 12.5% SDS-polyacrylamide gel, and proteins were detected using a Molecular imager (Bio-Rad). Two microliters of in vitro-translated proteins were loaded on a parallel gel as a control.

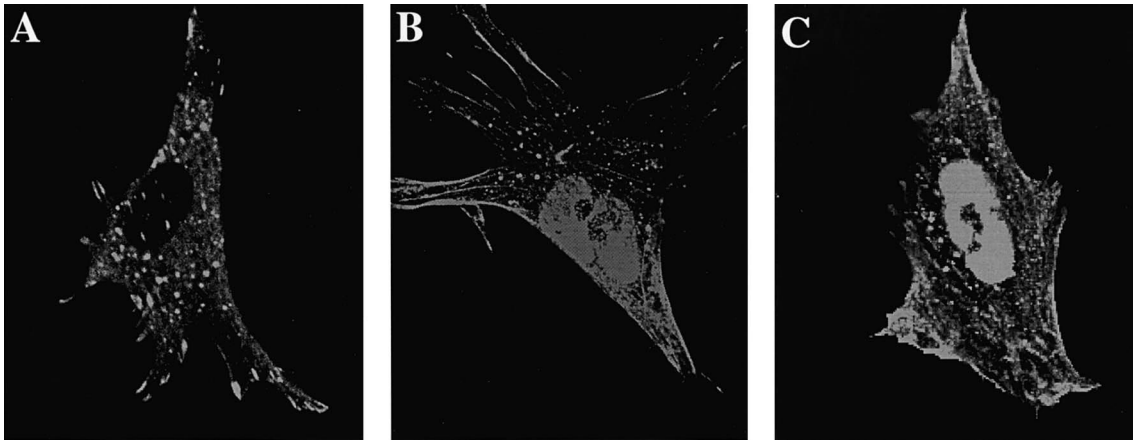


Fig. 1. Treatment of cells with leptomycin B causes Trip6 to accumulate in the nucleus. CEF were transfected with a FLAG-Trip6 expression vector and were analyzed by indirect immunofluorescence using an anti-FLAG primary antibody. (A) Untreated CEF. (B) CEF treated with leptomycin B for 15 min. (C) CEF treated with leptomycin B for 4 h.

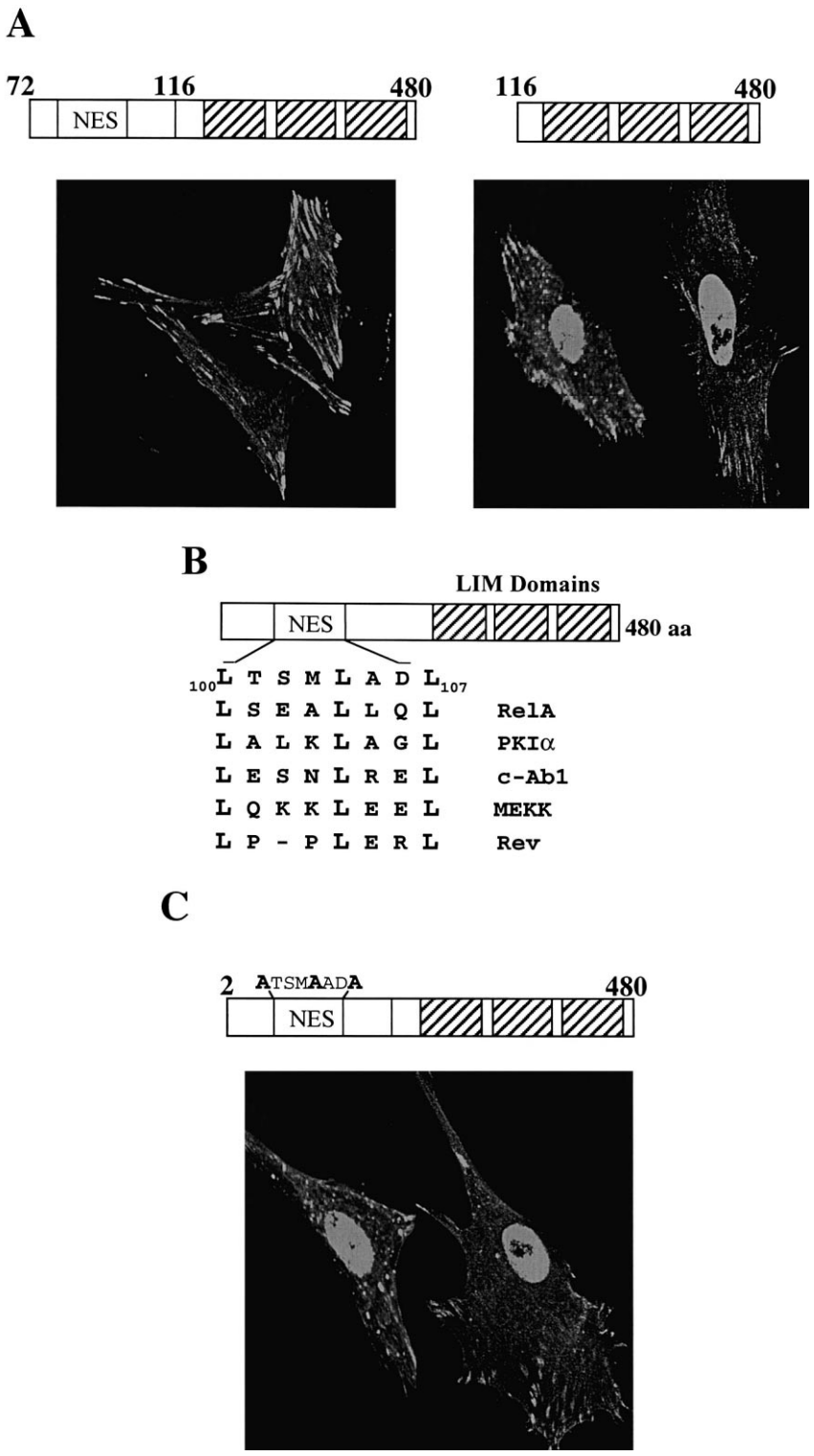

A
72
116
480
NES
116
480
B
LIM Domains
NES
480 aa
100L T S M L A D L107
L S E A L L Q L RelA
L A L K L A G L PKIα
L E S N L R E L c-Ab1
L Q K K L E E L MEKK
L P - P L E R L Rev
C
2 ATSMAADA
480
NES

Fig. 2

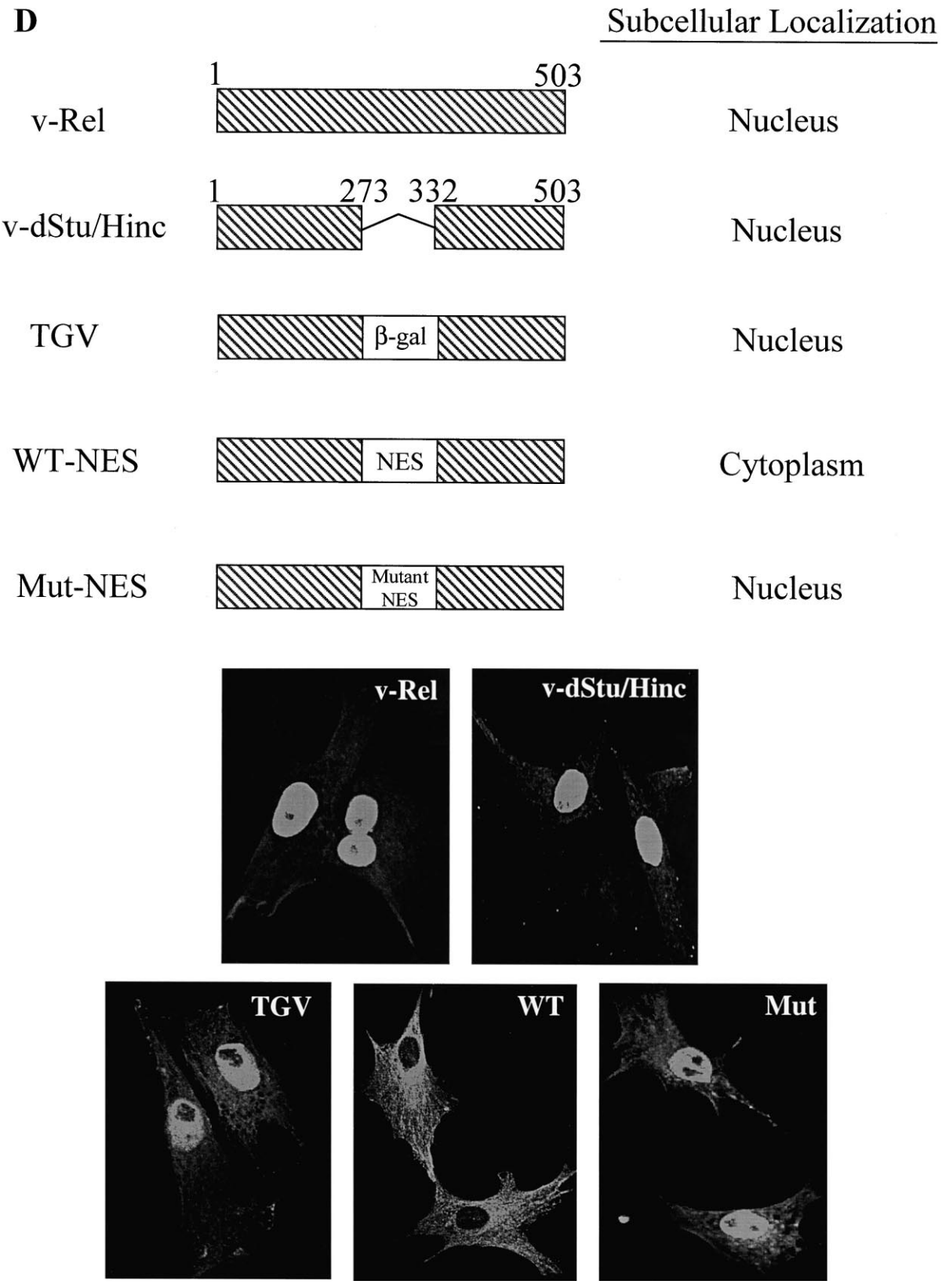

D
Subcellular Localization
1
503
v-Rel
Nucleus
1
273
332
503
v-dStu/Hinc
Nucleus
TGV
β-gal
Nucleus
WT-NES
NES
Cytoplasm
Mut-NES
Mutant NES
Nucleus
v-Rel
v-dStu/Hinc
TGV
WT
Mut

Fig. 2

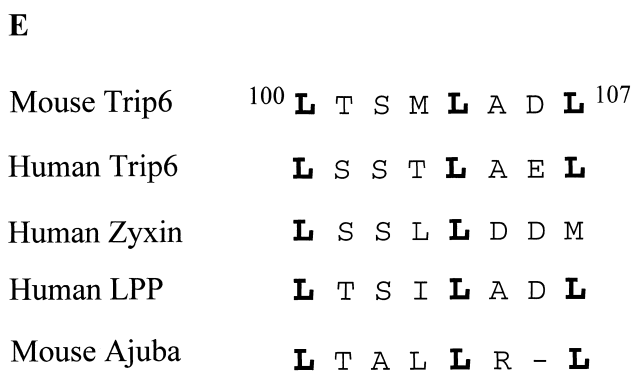


Fig. 2. Trip6 contains a leucine-rich NES between aa 100 and 107. (A) The subcellular localization of the indicated FLAG-tagged deletion mutants of Trip6 in transfected CEF was determined by indirect immunofluorescence using an anti-FLAG primary antiserum as in Fig. 1. Hatched boxes, LIM domains; numbers indicate Trip6 aa in the protein. (B) Comparison of NES of mouse Trip6 with other NESs. Essential leucine residues (L) are in larger font. (C) The indicated leucine residues of FLAG-Trip6 were mutated to alanine residues (bold A) and the subcellular location of the indicated protein was determined as in (A). (D) The subcellular localization of the indicated recombinant v-Rel proteins in CEF was determined by indirect immunofluorescence using an anti-v-Rel primary antiserum. (E) The Trip6 NES is aligned with the analogous sequences in zyxin, LPP, and Ajuba.

The generation of chicken spleen cells transformed by retroviral vector pMH-v-Rel-IRES-FLAG-ΔN-Trip6 has been described previously [7]. Eight milliliters of an exponentially growing culture of these suspension cells were pelleted and cells were then incubated in 150 μl of lysis buffer (0.2 M NaCl, 50 mM Tris, pH 7.5, 0.2 mM $ZnCl_2$, 0.5% NP-40, 0.5 mM DTT, 10% glycerol, 1% aprotinin (Sigma), 2 μg/ml leupeptin (Sigma)) on ice for 40 min. Samples were pelleted at top speed in a microcentrifuge for 10 min at 4°C, and the supernatant was then repelleted for 20 min at 4°C. 120 μl of supernatant was incubated with BD buffer containing DNA–cellulose, and beads were washed as described above. Samples were then boiled in 2×SDS sample buffer and resolved on 12.5% SDS-polyacrylamide gels. Fifteen microliters of cell lysate supernatant (described above) was loaded on the gel in parallel as a control. Western blotting was then performed using either anti-Rel or anti-FLAG antiserum as a primary antibody as described previously [6].

3. Results

3.1. Treatment of cells with leptomycin B causes Trip6 to accumulate in the nucleus

The Trip6-related proteins zyxin and LPP have been shown to shuttle between focal adhesion plaques and the nucleus [4,5]. For this reason, we were interested in determining whether Trip6, which is primarily located in adhesion plaques when overexpressed in CEF [6], also shuttles between cytoplasmic and nuclear compartments. Therefore, CEF transfected with a FLAG-Trip6 expression vector were treated with leptomycin B, which is an inhibitor of Crm1-dependent nuclear export [20,21]. The subcellular localization of FLAG-Trip6 was then analyzed by indirect immunofluorescence using an anti-FLAG primary antibody. Consistent with our previous results [6,7], over-expressed FLAG-Trip6 in untreated CEF resides primarily in focal adhesion plaques (Fig. 1A). However, upon treatment of cells with leptomycin B for as short as 15 min or as long as 4 h, the majority of FLAG-Trip6 was localized in the nucleus (Fig. 1B,C). These results suggest that Trip6 shuttles between cytoplasmic and nuclear compartments, and that the movement of Trip6 from the nucleus to the cytoplasm is dependent on Crm1, as it is blocked specifically by leptomycin B.

3.2. Trip6 contains a conserved NES between residues 100 and 107

We have previously shown that deletion of the N-terminal 115 aa of mouse Trip6 causes it to accumulate in the nucleus [6,7], suggesting that these sequences contain a NES. Therefore, we used three approaches to identify the Trip6 NES. First, deletion

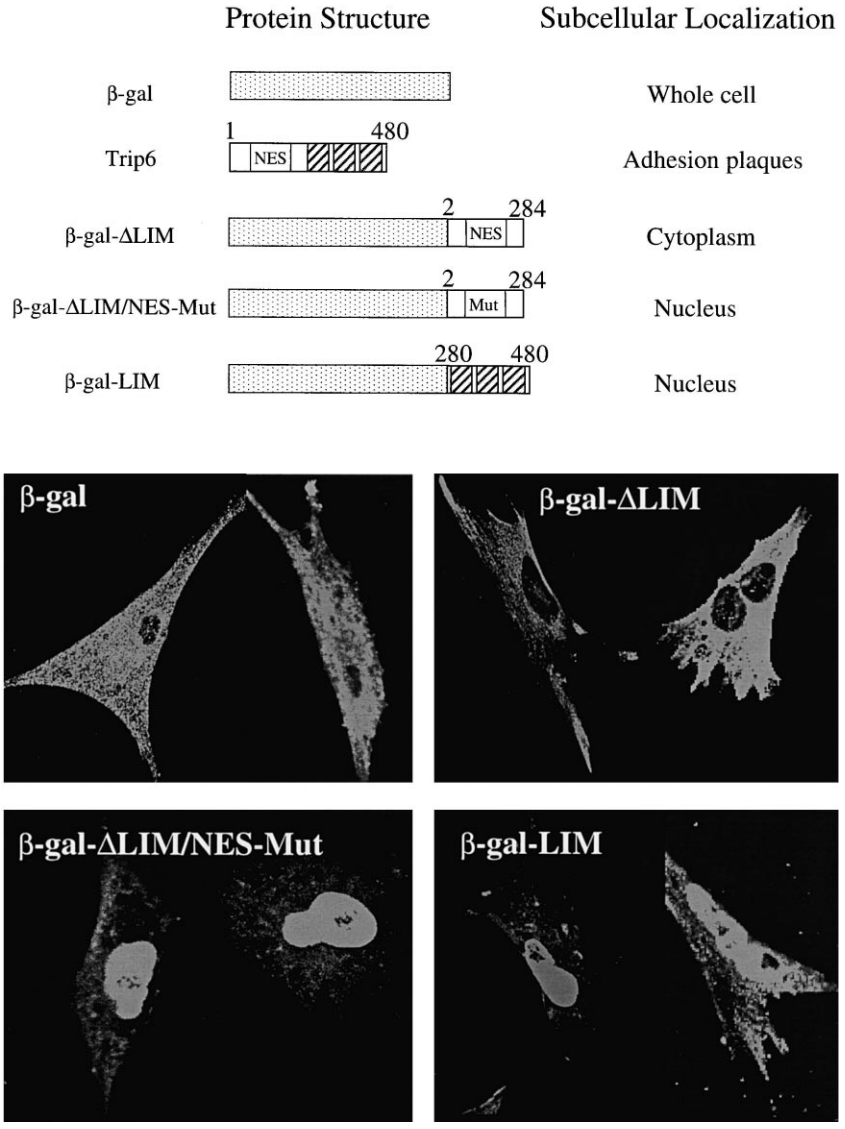


Fig. 3. N- and C-terminal sequences of Trip6 can direct β-gal to the nucleus. Indirect immunofluorescence (bottom) using an anti-β-gal primary antiserum of CEF transfected with expression vectors for the Trip6–β-gal fusion proteins shown at the top. The numbers above the figures refer to aa from mouse Trip6 included in each protein. Mut, mutant NES.

analysis indicated that the Trip6 NES was located between residues 72 and 115 of mouse Trip6, in that deletion of these sequences caused nuclear accumulation of Trip6 (Fig. 2A). Second, inspection of these sequences identified a leucine-rich sequence (Fig. 2B), which is characteristic of Crm1-dependent NESs. Third, conversion of the leucine residues within this putative NES to alanine residues caused FLAG-Trip6 to accumulate in the nucleus (Fig. 2C).

If these sequences in Trip6 were an independent NES, we reasoned that they should be able to export a heterologous protein from the nucleus. For these experiments we used a mutant of the v-Rel oncoprotein (v-dStu/Hinc), which is primarily located in the nucleus of CEF (Fig. 2D, panel v-dStu/Hinc) [15]. (This deletion mutant of v-Rel was used since it can conveniently accommodate insertions in the middle portion of the protein and it does not interact with cellular proteins that might affect localization of v-Rel [15].) Insertion of Trip6 aa 76 to 115 in the middle of the v-dStu/Hinc mutant caused this v-Rel protein to localize primarily to the cytoplasm

(Fig. 2D, panel WT). In contrast, addition of the same Trip6 sequences containing the mutant NES or addition of sequences from control protein (β-gal) did not affect the primarily nuclear localization of v-dStu-Hinc (Fig. 2D, panels Mut and TGV).

Taken together, the results in this section indicate that Trip6 has a leucine-rich NES. Notably, this leucine-rich sequence is conserved in the related proteins zyxin, LPP and Ajuba (Fig. 2E).

3.3. The N- and C-terminal regions of Trip6 have nuclear targeting functions that can enhance nuclear accumulation of β-gal

Because Trip6 can enter the nucleus after treatment with leptomycin B and has an NES, we suspected that Trip6 also had sequences that could direct it to the nucleus. To determine whether Trip6 has a nuclear targeting function, we fused either the N- or C-terminal halves of Trip6 to β-gal and monitored the subcellular localization of the proteins by indirect immunofluorescence using an anti-β-gal primary antibody. β-gal by itself is evenly distributed between the cytoplasm and the nucleus in CEF (Fig. 3, panel β-gal). Fusion of the C-terminal half (aa 280–480) of Trip6 to the C-terminus of β-gal resulted in a β-gal fusion protein that was located in the nucleus (Fig. 3, panel β-gal-LIM). This result indicates that Trip6 aa 280–480, which contain the three LIM domains, contain a sequence capable of targeting a heterologous protein to the nucleus.

We also fused sequences in the N-terminal half of Trip6 (aa 2–282) to the C-terminus of β-gal. This protein was located exclusively in the cytoplasm (Fig. 3, panel β-gal-ΔLIM), consistent with it containing a functional NES as determined above (see Fig. 2). However, if these N-terminal Trip6 sequences contained a mutated and non-functional NES, the β-gal fusion protein was now located in the nucleus. This indicates that the N-terminal region of Trip6 also contains a nuclear targeting sequence, whose activity is evident only upon disabling of the NES.

3.4. Trip6 has multiple transactivation domains

We have previously shown that C-terminal sequences of mouse or human Trip6, including the LIM domains, can activate transcription in yeast and CEF when fused to the GAL4 DNA-binding domain [6,7]. As shown in Fig. 4A, GAL4 fusion proteins containing either full-length Trip6 or the related protein zyxin can also readily activate transcription in CEF. To map the sequences in Trip6 responsible for

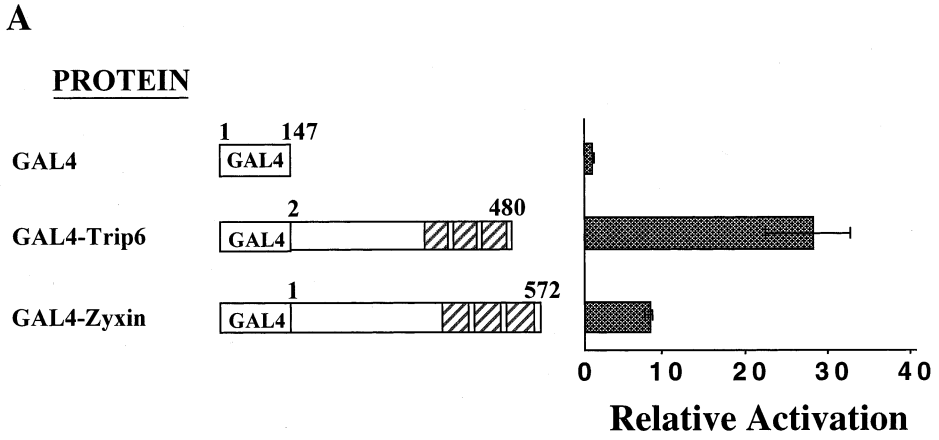
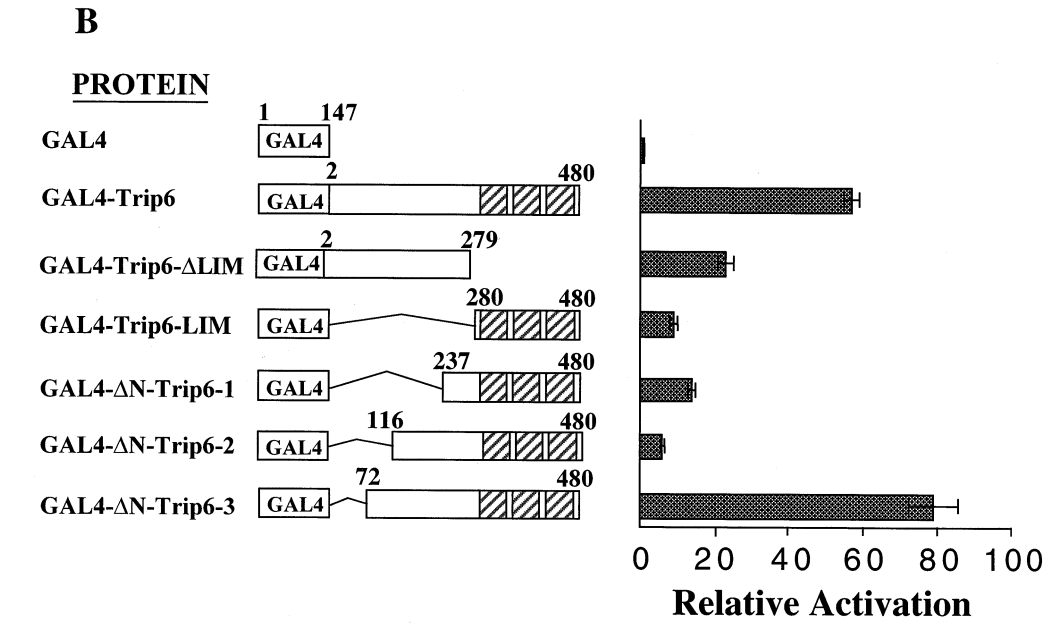
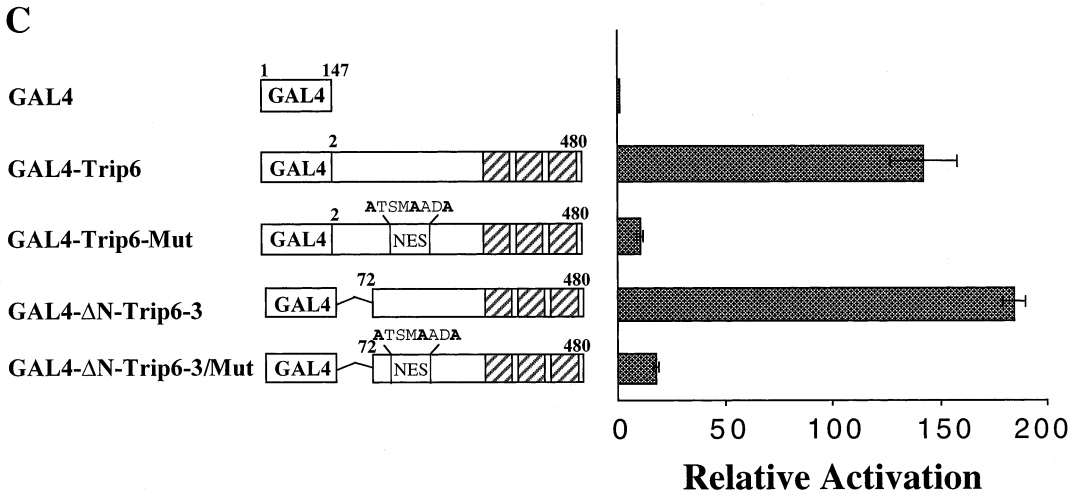


Fig. 4. Trip6 contains multiple transactivation domains. The transcriptional activating ability of the indicated GAL4-fusion proteins was determined by CAT reporter gene assays in CEF. Fold activation is relative to the level of acetylation seen with GAL4 alone (1.0). (A) GAL4–Trip6 and GAL4–Zyxin can activate transcription. (B) Transactivation by the indicated Trip6 deletion mutants was determined. (C) Mutation of the Trip6 NES reduces transactivation by GAL4–Trip6 proteins. (D) Comparison of subcellular location and transcription activation by Trip6 NES mutants. Subcellular localization was performed by indirect immunofluorescence on full-length Trip6 proteins as described for Fig. 2. Transactivation was determined using GAL4-full-length Trip6 fusion proteins as in (C). (E) Indirect immunofluorescence using an anti-GAL4 primary antibody of CEF transfected with expression vectors for the indicated proteins.





D

	Location	Trans-activation
LTSMLADL (NES)	Adhesion Plaques	18.4
ATSMAADA	Nucleus	2.7
ATSMAADL	Nucleus	4.1
LTSMLADA	Nucleus	2.9

Fig. 4 (*continued*)

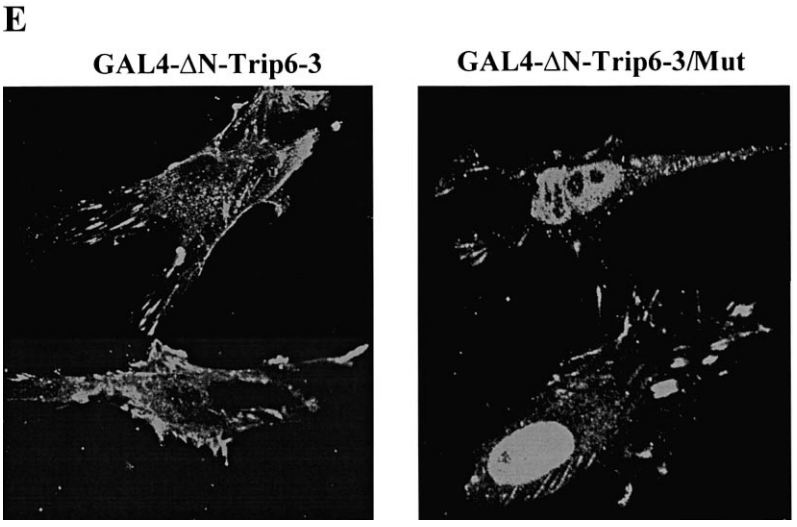


Fig. 4 (*continued*)

transcription activation, we performed GAL4-site CAT reporter gene assays in CEF with a series of GAL4–Trip6 fusion proteins (Fig. 4B). This analysis shows that sequences from both the N- and C-terminal halves of Trip6 contribute to its transactivation ability. The deletion mutants indicate that Trip6 contains one transactivation domain in the C-terminal LIM domain region (aa 280–480) and a second transactivation domain between residues 72 and 115.

Because the N-terminal transactivation region (aa 72–115) in Trip6 also contains the NES, we wished to determine whether the NES was functionally distinct from the transactivation activity. Therefore, we assayed the ability of the Trip6 NES Leu-to-Ala triple mutant to activate transcription. As shown in Fig. 4C, mutation of all Leu residues within the NES greatly reduced the ability of GAL4–Trip6 fusion proteins to activate transcription (compare full-length and ΔN mutants with the corresponding wild-type Trip6 proteins, Fig. 4C). In addition, single or double Leu-to-Ala mutations within the NES resulted in both nuclear accumulation and reduced transactivation (Fig. 4D).

Because the NES mutations altered a function involved in subcellular localization, we were interested in comparing the subcellular localization of wild-type and NES mutant GAL4–Trip6 proteins. Wild-type GAL4–Trip6 shows a primarily cytoplasmic staining pattern, whereas the NES mutant GAL4–Trip6 protein is primarily in the nucleus (Fig. 4E). Thus, wild-type GAL4–Trip6 activates transactivation more strongly than NES mutant GAL4–Trip6 proteins even though there is less wild-type GAL4–Trip6 protein in the nucleus. Taken together, the results in this section demonstrate that the same Leu-to-Ala mutations that alter the Trip6 NES function reduce transactivation, suggesting that the NES and transactivation sequences of Trip6 within aa 72 and 115 overlap.

3.5. Trip6 does not bind to DNA–cellulose in vitro

Because of the nuclear functions of Trip6 and the fact that LIM domains are often associated with DNA-binding proteins [7,9,11] we next determined whether Trip6 could bind DNA. In these experiments, in vitro-translated Trip6 or Trip6 isolated from v-Rel-transformed spleen cells was analyzed for its ability to bind to DNA–cellulose. Because the binding of the related LIM domain protein Hic-5 is dependent on zinc [3], zinc was included in all experiments. As shown in Fig. 5A, in vitro-translated full-length Trip6 or ΔN-Trip6 (aa 116–480 of Trip6) did not bind to DNA–cellulose. As controls in these experiments, the v-Rel transcription factor did bind to DNA (last lane in right panel of Fig. 5A), but a v-Rel mutant (v-dStu/Hinc, see Fig. 2D) that cannot bind to κB sites and the plasma membrane protein c-Src did not bind to DNA (Fig. 5A right panel). Similarly, when isolated from v-Rel-transformed spleen cells, FLAG-ΔNTrip6 did not bind

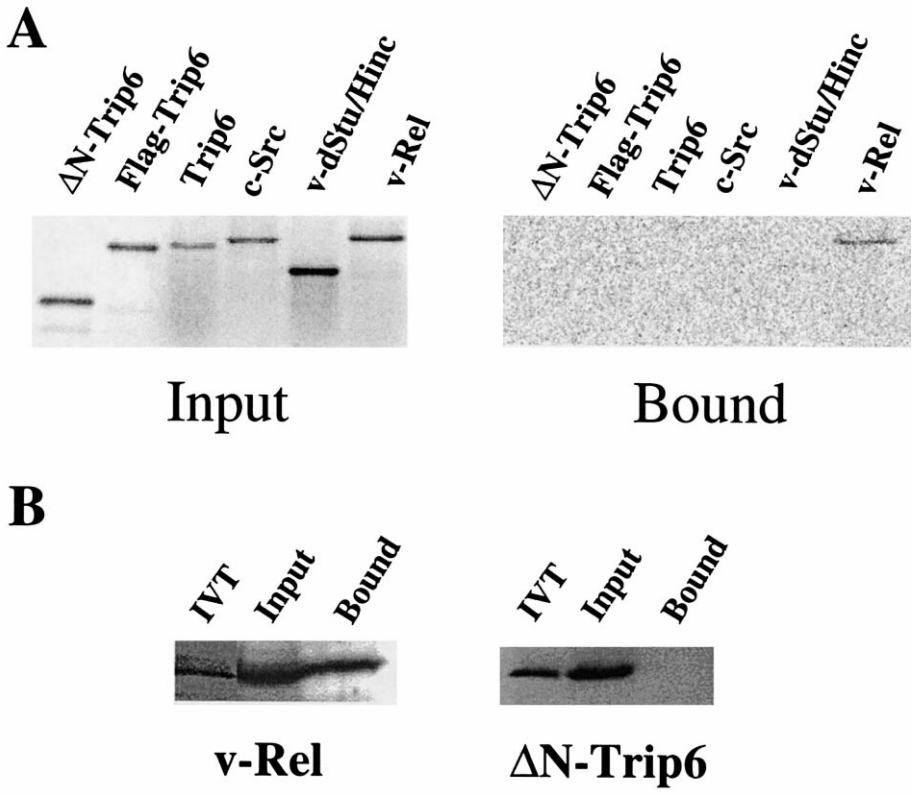


Fig. 5. In vitro- and in vivo-synthesized Trip6 does not bind to DNA–cellulose. (A) The indicated proteins were translated in vitro in the presence of Tran^{35}S-label (Input) and were incubated with DNA–cellulose (Bound) as described in Section 2.3. Samples were then detected by molecular imaging. The input lanes have approximately 1/3 to 1/5 the amount of protein as in the bound lanes. (B) Lysates from chicken spleen cells transformed with an expression vector for v-Rel and FLAG-ΔN-Trip6 (lanes 2 and 3 in each panel) were analyzed directly (Input) or were incubated with DNA–cellulose (Bound). Western blotting was performed with anti-v-Rel antiserum (left panel) or anti-FLAG antiserum (right panel). In each case, the respective in vitro-translated proteins (IVT) were used as size controls.

to DNA, whereas v-Rel did (Fig. 5B). These results indicate that, at least under the conditions used here, neither full-length Trip6 nor the LIM domain region of Trip6 can bind to DNA–cellulose.

4. Discussion

In this report, we provide extensive evidence that LIM domain protein Trip6 shuttles between focal adhesion plaques and the nucleus. That is, treatment of cells with leptomycin B, an inhibitor of Crm1-dependent nuclear export, results in nuclear accumulation of Trip6, and Trip6 has an independent NES. In addition, N- and C-terminal sequences of Trip6, including the LIM domains, have a nuclear targeting signal. Moreover, we show that Trip6 has multiple transactivation domains, and that mutations that abolish the NES function also lessen transactivation by Trip6.

Although Trip6 and the related proteins zyxin and LPP have all been shown to shuttle between focal adhesion plaques and the nucleus, these proteins appear to largely reside in adhesion plaques in the steady-state [5–7,9]. For example, at any given moment, very little Trip6 appears in the nucleus in control cells overexpressing Trip6 (see Fig. 1A). Nevertheless, treatment of CEF with leptomycin B for as little as 15 min results in an almost exclusively nuclear localization of Trip6. One interpretation of these results is that Trip6 is very rapidly exported from the nucleus once it enters it. Alternatively, the focal adhesion plaque localization of Trip6 is dependent on a leptomycin B sensitive function or protein,

which, when inhibited, causes Trip6 to rapidly enter the nucleus.

Our results identify the Trip6 NES as being part of a leucine-rich sequence from aa 100 to 107, which is analogous to the NESs that were identified in LPP and Ajuba [5,8]. Curiously, a different leucine-rich sequence was identified as an NES in the highly related protein zyxin [4], even though both leucine-rich sequences are highly related in Trip6, LPP and zyxin [6]. Our identification of the Trip6 NES is unambiguous: that is, deletion or mutation of this sequence sequesters Trip6 in the nucleus and this NES can independently export a heterologous protein (v-Rel) from the nucleus (Fig. 2). Moreover, deletion of the zyxin-like NES from Trip6 does not alter its focal adhesion plaques localization (Wang and Gilmore, unpublished results). Thus, the reason for the difference in function of these leucine-rich NESs between Trip6 and LPP versus zyxin is not clear.

We have also shown that sequences of Trip6, including the LIM domains, can direct cytoplasmic β-gal to the nucleus. Inspection of the Trip6 sequences reveals no obvious consensus (basic) nuclear targeting sequence [22]. Thus, either Trip6 has an unconventional nuclear localization sequence or, for example, the LIM domains interact with a protein that brings these sequences to the nucleus. Of note, armadillo repeat domains, also protein–protein interaction domains, have been shown to participate directly in nuclear localization of β-catenin [23,24] and possibly the APC tumor suppressor protein [25].

Our results have also identified (at least) two transactivation domains in Trip6: one between aa 72 and 115 and one including the C-terminal LIM domains. The transactivation domain between 72 and 115 appears to overlap with the Trip6 NES in that Leu-to-Ala mutations that abolish the nuclear export function also greatly lessen the transactivation function. Alternatively, nuclear export of Trip6 is required for a nearby transactivation domain to function maximally. In either case, to our knowledge, this is the first identification of an NES that is part of or required for a transactivation domain.

It is still not clear what relevance nuclear localization or transactivation has for the function of Trip6. In that we have shown that Trip6 does not bind to DNA–cellulose (Fig. 5), it appears unlikely that Trip6 functions as a direct activator of transcription. We have previously shown that a nuclear-localized form of Trip6 can act as a co-activator for the v-Rel transcription factor [7]. In addition, Trip6 can interact with thyroid hormone receptor, a nuclear localized sterol receptor, in a hormone-dependent manner [11]. Of note, JAB1, which was originally identified as a co-activator of the c-Jun transcription factor, is co-localized with integrin LFA-1 at the cell membrane, but upon activation of LFA-1 a fraction of JAB1 enters the nucleus [26].

However, no cellular signal has been identified that can induce nuclear localization of Trip6, nor the related proteins zyxin, LPP, or Ajuba. For example, activation of focal adhesion kinase (FAK) by v-Src does not induce nuclear translocation of Trip6 nor does treatment of cells with cytochalasin D, an actin filament disrupting reagent, or okadaic acid, a Ser/Thr phosphatase inhibitor, induce its nuclear translocation (Wang and Gilmore, unpublished results).

Although zyxin, Trip6, LPP, Ajuba, and Hic-5 are highly related LIM domain proteins, which largely reside in adhesion plaques but can enter the nucleus and can activate transcription, these proteins clearly have differences. For example, although Ajuba can enter the nucleus, it appears to be largely cytosolic [8] rather than localized in adhesion plaques like other members of this family. Similarly, although Hic-5 has been shown to bind to DNA [3], the other members of this family have not. As described above, the sequences that act as NESs for zyxin and Trip6 may differ. Furthermore, although zyxin and LPP have been shown to bind to the VASP/Ena protein [5,10], which is likely to be involved in the regulation of cell migration [27], Trip6 does not appear to have a VASP-Ena binding domain. Lastly, nuclear-localized Ajuba and Trip6 proteins can promote embryonic carcinoma cell differentiation, but zyxin cannot [8]. Taken together, these results indicate that these related LIM domain proteins have distinct functions. It will be of interest to determine the effect of disruption of the genes encoding these proteins in mice and the effect of their overexpression in tissue culture cells.

Acknowledgements

This work was supported by Public Health Service

Grant CA47763 from the National Cancer Institute (to T.D.G.). T.D.G. and Y.W. were partially supported by a grant from the Tobacco Research Council, Inc. We thank Minoru Yoshida for the kind gift of leptomycin B, G.S. Martin for the c-Src cDNA, Jason Baum for help in the construction of pSG-zyxin, and Mary Beckerle for the zyxin cDNA and helpful discussions.

References

[1] I. Bach, Mech. Dev. 91 (2000) 5–17.
[2] I.B. Dawid, J.J. Breen, R. Toyama, Trends Genet. 14 (1998) 156–162.
[3] N. Nishiya, H. Sabe, K. Nose, M. Shibanuma, Nucleic Acids Res. 26 (1998) 4267–4273.
[4] D.A. Nix, M.C. Beckerle, J. Cell Biol. 138 (1997) 1139–1147.
[5] M.M. Petit, J. Fradelizi, R.M. Golsteyn, T.A. Ayoubi, B. Menichi, D. Louvard, W.J. Van de Ven, E. Friederich, Mol. Biol. Cell 11 (2000) 117–129.
[6] Y. Wang, J.E. Dooher, M. Koedood Zhao, T.D. Gilmore, Gene 234 (1999) 403–409.
[7] M. Koedood Zhao, Y. Wang, K. Murphy, J. Yi, M.C. Beckerle, T.D. Gilmore, Gene Expr. 8 (1999) 207–217.
[8] J. Kanungo, S.J. Pratt, H. Marie, G.D. Longmore, Mol. Biol. Cell 11 (2000) 3299–3313.
[9] M.C. Beckerle, Bioessays 19 (1997) 949–957.
[10] B. Drees, E. Friederich, J. Fradelizi, D. Louvard, M.C. Beckerle, R.M. Golsteyn, J. Biol. Chem. 275 (2000) 22503–22511.
[11] J.W. Lee, H.S. Choi, J. Gyuris, R. Brent, D.D. Moore, Mol. Endocrinol. 9 (1995) 243–254.
[12] E. Cuppen, M. van Ham, D.G. Wansink, A. de Leeuw, B. Wieringa, W. Hendriks, Eur. J. Cell Biol. 79 (2000) 283–293.
[13] K.K. Murthy, K. Clark, Y. Fortin, S.H. Shen, D. Banville, J. Biol. Chem. 274 (1999) 20679–20687.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[15] T.D. Gilmore, H.M. Temin, J. Virol. 62 (1988) 703–714.
[16] S. Sif, A.J. Capobianco, T.D. Gilmore, Oncogene 8 (1993) 2501–2509.
[17] I. Sadowski, M. Ptashne, Nucleic Acids Res. 17 (1989) 7539.
[18] J.-C. Epinat, D. Kazandjian, D.D. Harkness, S. Petros, J. Dave, D.W. White, T.D. Gilmore, Oncogene 19 (2000) 599–607.
[19] A.J. Capobianco, T.D. Gilmore, Oncogene 6 (1991) 2203–2210.
[20] M. Fornerod, M. Ohno, M. Yoshida, I.W. Mattaj, Cell 90 (1997) 1051–1060.
[21] N. Kudo, B. Wolff, T. Sekimoto, E.P. Schreiner, Y. Yoneda, M. Yanagida, S. Horinouchi, M. Yoshida, Exp. Cell Res. 242 (1998) 540–547.
[22] D.A. Jans, S. Hubner, Physiol. Rev. 76 (1996) 651–685.
[23] F. Fagotto, U. Gluck, B.M. Gumbiner, Curr. Biol. 8 (1998) 181–190.
[24] N. Funayama, F. Fagotto, P. McCrea, B.M. Gumbiner, J. Cell Biol. 128 (1995) 959–968.
[25] R. Rosin-Arbesfeld, F. Townsley, M. Bienz, Nature 406 (2000) 1009–1012.
[26] E. Bianchi, S. Denti, A. Granata, G. Bossi, J. Geginat, A. Villa, L. Rogge, R. Pardi, Nature 404 (2000) 617–621.
[27] L.M. Machesky, Cell 101 (2000) 685–688.