Identification of novel cellular proteins that bind to the LC8 dynein light chain using a pepscan technique

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Abstract Dynein is a minus end-directed microtubule motor that serves multiple cellular functions. We have performed a fine mapping of the 8 kDa dynein light chain (LC8) binding sites throughout the development of a library of consecutive synthetic dodecapeptides covering the amino acid sequences of the various proteins known to interact with this dynein member according to the yeast two hybrid system. Two different consensus sequences were identified: GIQVD present in nNOS, in DNA cytosine methyl transferase and also in GKAP, where it is present twice in the protein sequence. The other LC8 binding motif is KSTQT, present in Bim, dynein heavy chain, Kid-1, protein 4 and also in swallow. Interestingly, this KSTQT motif is also present in several viruses known to associate with microtubules during retrograde transport from the plasma membrane to the nucleus during viral infection. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Migration; Microtubule; Dynein; Nitric oxide; Yeast two hybrid; Pepscan

1. Introduction

Dyneins are large multi-component, microtubule-based molecular motors that generate force towards the minus end of microtubules and are involved in several cellular processes including retrograde transport in axons, ciliary/flagellar beating, vesicular transport, movement of endosomes and lysosomes and positioning of the mitotic spindle [1–3]. From a structural and functional point of view, the two major classes of dyneins are the axonemal dyneins involved in flagellar and ciliary movement, and the cytoplasmic dyneins. In generic terms, cytoplasmic dyneins are constructed around one or more heavy chains (HCs), each of which forms a multilobed globular head structure where the ATP hydrolysis responsible for the microtubule motor activity takes place. These HCs are associated to several (probably two) copies of a 74 kDa intermediate chain (IC), four light intermediate chains (LICs) of 50–60 kDa, and several light chains (LCs) with molecular masses lower than 22 kDa. In the case of cytoplasmic dynein, this global association of several polypeptide chains results in a total molecular mass of ~1.25 MDa [1–3].

Recently, King and coworkers have identified and characterised in detail the three LC components of 8 kDa, 14 kDa and 22 kDa within cytoplasmic dynein [4–7]. The 8 kDa dynein LC (LC8, actual mass 10 kDa) was first identified as an integral component of the *Chlamydomonas reinhardtii* outer dynein arm, in association with the ICs at the base of the soluble particle [8]. To date, at least 10 different proteins under study in independent laboratories were used as bait in a yeast two hybrid experiment and retrieved LC8 as a high affinity interacting protein. The cellular LC8-associated proteins include the N-terminus region of neuronal nitric oxide synthase (nNOS) [9], IκBα [10], the renal transcription factor Kid-1 (Dr Ralph Witzgall, University of Heidelberg, personal communication), the proapoptotic member of the Bcl-2 family Bim [11], the product of the *Drosophila* swallow gene [12], myosin V [13] and a guanylate kinase domain-associated protein (GKAP) [13].

We describe herein a new approach that allows a fast analysis of the LC8 binding sites to be performed within sequences of proteins that associate with microtubules and become transported in a retrograde manner. Likewise, we have also screened other protein sequences suggested to be interacting with LC8 according to biochemical and structural data.

2. Materials and methods

2.1. Materials

Escherichia coli BL21 DE3 competent cells were from Novagen. Antibodies against the hexahistidine tag were purchased from Sigma. The Ni–nitrilotriacetic acid resin was from Qiagen. ECL reagents were obtained from Amersham-Pharmacia Biotech. Photography film was purchased from Kodak. All the other reagents were from Sigma.

2.2. Peptide synthesis

Overlapping dodecapeptides for mapping studies were prepared by automated spot synthesis (Abimed, Langenfeld, Germany) onto an amino-derivatised cellulose membrane, immobilised by their C-termini via a polyethylene glycol spacer, and N-terminal acetylated. We used a sequential displacement of two amino acids during the synthesis, which leads to an overlap of 10 residues between each two consecutive dodecapeptides, an 8 residue overlap among three consecutive do-

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Abbreviations: LC8, 8 kDa dynein light chain; GKAP, guanylate kinase domain-associated protein; HC, heavy chain; IC, intermediate chain; KRAB, Krüppel-associated box; LC, light chain; LIC, light intermediate chain; nNOS, neuronal nitric oxide synthase

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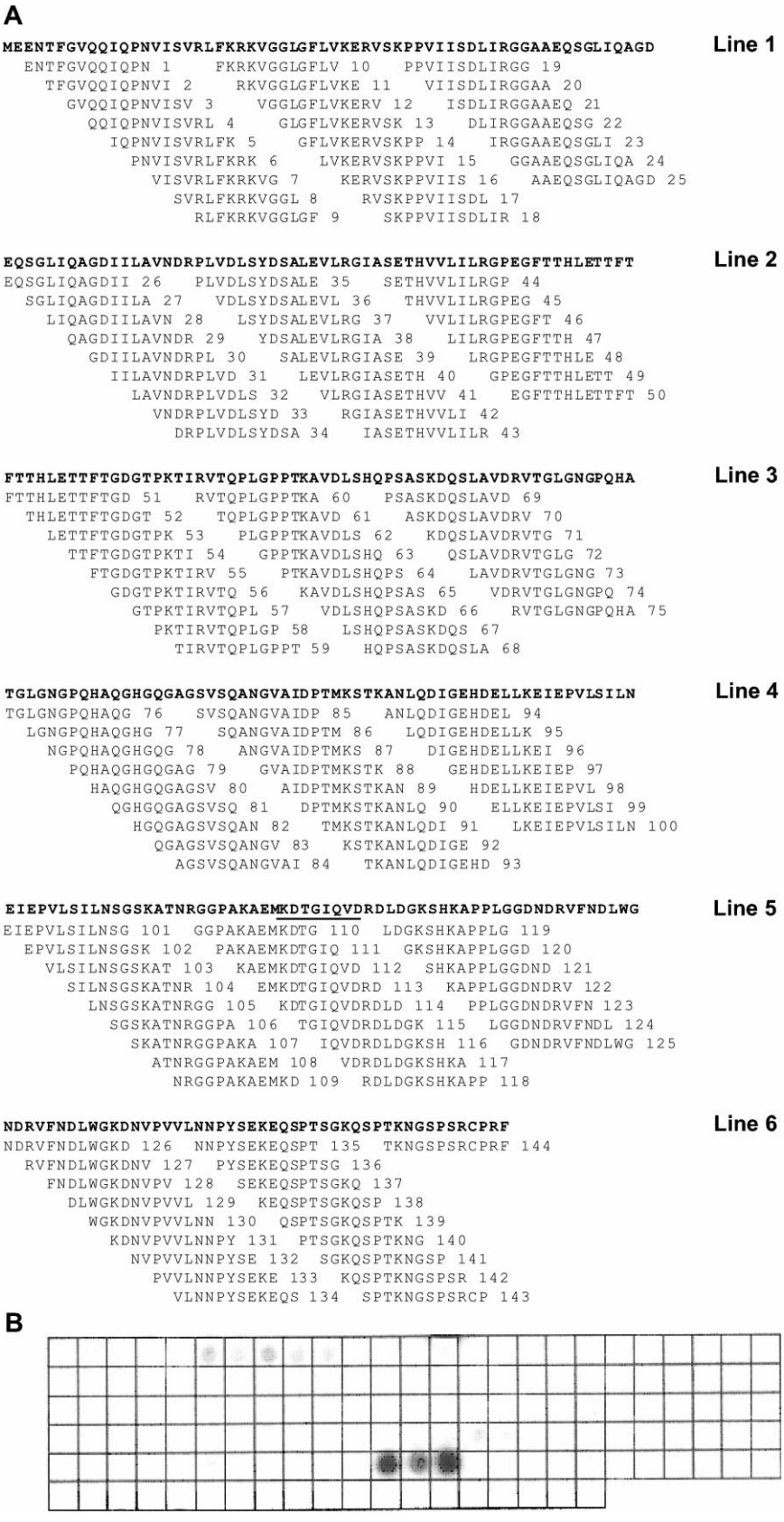

Fig. 1. Binding of LC8 to nNOS. Recombinant LC8 was incubated with a cellulose membrane bearing 144 spots with synthetic dodecapeptides that cover the first 300 amino acids of the protein (A). 25 dodecapeptides were synthesised on each line (except line 6 with 19 peptides). The membrane was developed by ECL after incubation with a peroxidase-labelled anti-hexahistidine tag antibody (B). The underlined sequence (KDTGIQVD) on line 5 indicates the overlap among peptides 112, 113 and 114.

decapeptides, a 6 residue overlap among four consecutive dodecapeptides, and so on.

2.3. Expression and purification of the recombinant LC8 proteins

The pET-LC8 plasmid was used to routinely transform BL21 DE3 cells, and LC8 was expressed and purified as previously reported [14]. In summary, a fresh colony was picked and used to inoculate 2 ml of LB medium that was grown overnight at 37°C. This culture was used to initiate a larger culture of 1 l of 2×YT medium that was grown in 2 l flasks at 230 rpm at 25°C. Purified protein eluted from the Ni–nitrilotriacetic acid column was dialysed against 100 mM $(NH_4)HCO_3$ and frozen in liquid nitrogen. Protein storage was performed at −20°C in small aliquots in order to avoid repetitive freeze–thawing.

2.4. Recombinant LC8 binding assays on cellulose-bound peptides

The cellulose membranes were coated with 1% non-fat dried milk in

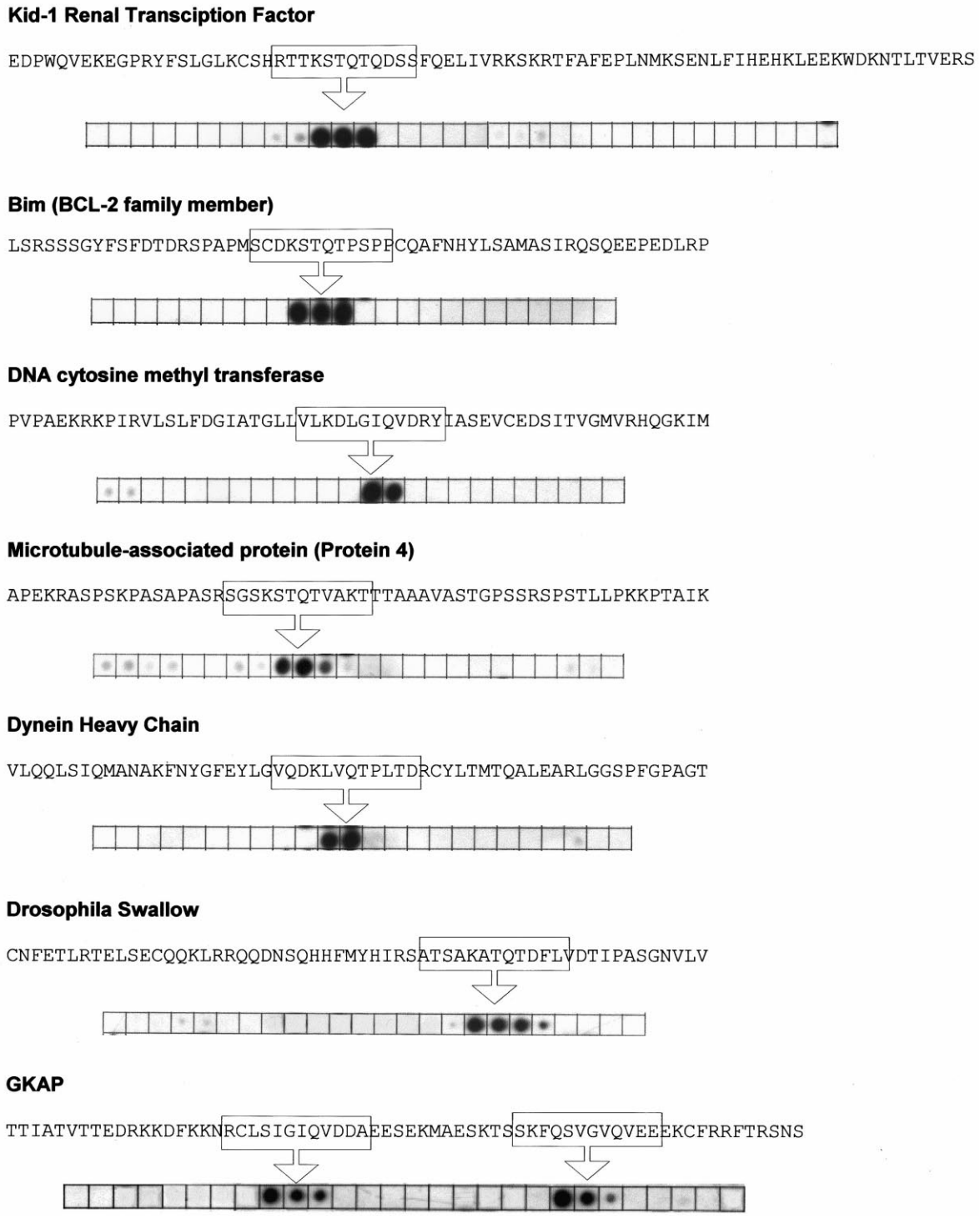

Fig. 2. Binding of LC8 to various cellular targets. Recombinant LC8 was incubated with cellulose membranes bearing spots with synthetic dodecapeptides that cover the indicated sequences. Each spot covers a sequence with two amino acids displaced compared to the previous sequence. Selected positive spots are boxed and correlated with their corresponding sequence. The membrane was developed by ECL after incubation with a peroxidase-labelled anti-hexahistidine tag antibody.

TBS (50 mM Tris, pH 7.0, 137 mM NaCl, 2.7 mM KCl) for 4 h at room temperature. Incubation with the recombinant LC8 (0.13 μM) was done overnight at room temperature. Three washes (25 ml each) were performed with TBS–Tween 20 (0.05%). Subsequently, the membrane was incubated for 2 h at room temperature with a commercial antibody against the hexahistidine tag present in the recombinant protein (1:100 000 dilution in TBS). Three additional 10 min washes were performed with TBS–Tween 20 (0.05%), followed by three more 10 min washes with TBS alone. Development of the membrane was performed by ECL following the manufacturer's instructions. The quantification of the intensity of each spot was performed utilising a UVI-tec digital image analyser (UVItec, Cambridge, UK) and the software UVIband V97. In every case, spots corresponding to the dodecapeptides of the various proteins synthesised onto the same membrane were compared with each other. Controls performed with the antibody in the absence of recombinant LC8 were performed in every case, in order to subtract the non-specific binding due to the reactivity of the antibody against certain synthetic dodecapeptides.

3. Results

3.1. Binding of LC8 to nNOS

Since a crystallographic, three-dimensional structure of a peptide of nNOS bound to LC8 is available [15], we decided to validate our methodology covering a large portion of nNOS in the form of overlapping synthetic dodecapeptides and performing a binding assay to recombinant LC8 (Fig. 1). Development was performed using a peroxidase-labelled antibody that recognises the hexahistidine tag present in the recombinant LC8. Binding of LC8 to peptides 112, 113 and 114 reveals that the common amino acid sequence to these three peptides is the KDTGIQVD motif, in accordance with the yeast two hybrid and crystallographic data [9,15]. The recognition of LC8 toward peptides that possess this binding motif is exquisite, since no other dodecapeptide present in the membrane displays any comparable reactivity. Accordingly, we undertook the analysis of protein sequences known to interact to LC8 according to previous yeast two hybrid information, as well as several proteins known to be associated to microtubules.

3.2. Binding of LC8 to cellular targets

In order to perform a fine mapping of the recognition properties of LC8 towards other cellular proteins, we screened fragments of 13 different polypeptides ranging in size from 58 residues (in most of the cases, 24 spots) to 184 residues (myosin V). Seven of them (Kid-1, Bim, DNA cytosine methyl transferase, protein 4, dynein HC, swallow and GKAP) returned a positive interaction which converge at two to four consecutive spots (Fig. 2). In every case, a clear, distinct pattern of interaction was observed. Since two consecutive overlapping peptides possess 10 residues in common, and three consecutive overlapping peptides possess eight residues in common, we were able to map down the binding motifs to the minimal recognition sequence. This recognition motifs fall into two distinct categories, a GIQVD motif and a KSTQT motif (Table 1), with all the positive sequences falling into one of the two groups.

The GIQVD motif accepts another hydrophobic β-branched residue in place of the Ile residue at position two. In a similar fashion, a Glu residue can substitute for an Asp residue of position five. However, the Gly, Gln and Val residues are invariably present in all cases at positions one, three and four. The KSTQT motif also accept some conservative substitutions on its sequence, although the Lys, Gln and Thr residues remain mostly invariant. Position two can tolerate residues such as Ser, Ala, Leu and Gln, whereas at position three only residues such as Thr, Ser and Val are observed.

Large regions of six additional proteins failed to show any positive interaction according to the pepscan technique. These included IκBα and the actin-based molecular motor myosin V, in spite of the fact that both of them reportedly do so in the yeast two hybrid system [10,13]. Thus, the pepscan technique does not account for the interaction between these proteins and LC8. The other four proteins that failed to give a positive interaction were known to associate with microtubules (although their interaction with LC8 has not been reported) in addition to having polypeptide sequences that were somehow similar to the KSTQT or KDTGIQVD motifs. These were dynamin (with a DSWLQVQ stretch), myosin HC (KDTQLQLDD), AMP kinase (KDTGISCDPA) and β-tubulin (GDSDLQLDR).

3.3. Binding of LC8 to viral targets

Recently, it has been shown that rabies virus and its homologue, Mokola virus, bind to LC8 through their P protein, and both proteins can be co-immunoprecipitated in infected cells [16,17]. On the other hand, African Swine Fever virus uses a protein called p54, essential during the first infective steps to bind to LC8, and both proteins co-localise at the microtubular organisation centre during viral infection [18]. This discovery that several viruses are transported in a retrograde manner toward the nucleus bound to the dynein multiprotein complex led us to consider the analysis of sequences corresponding to these three viral proteins (Fig. 3). By means of the pepscan technique, we could conclude that the three viral proteins resulted in positive binding to LC8, and exhibit binding sequences that fall into the KSTQT motif (Table 1, Fig. 3).

3.4. Binding of LC8 to the KSTQT motif positioned in different sequence environments

A fast inspection of any eukaryotic sequence database reveals that there are hundreds of proteins that display either the KSTQT or the KDGIQVDR motifs, and just a small

Table 1
Summary of the sequences identified in proteins that interact with LC8

GIQVD motif	
KDT GIQVDR	rat nNOS
KDM GIQVDR	human nNOS[a]
RDT GVQVDR	rabbit nNOS[a]
KDL GIQVDR	DNA cytosine methyl transferase
LSI GIQVDD	GKAP
QSV GVQVEE	GKAP
GIQVD	general consensus
KSTQT motif	
CD KSTQTPS	Bim
TT KSTQTQD	Kid-1
SA KATQTDF	swallow
GS KSTQTVA	microtubule-associated protein
QD KLVQTPL	dynein HC
ED KSTQTPE	Mokola virus
ED KSTQTTS	Rabies virus
QN TASQTMS	African swine virus
KSTQT	general consensus

[a]The human and rabbit sequences of nNOS are depicted here in order to show the non-identical residues that can be accommodated flanking the GIQVD motif.

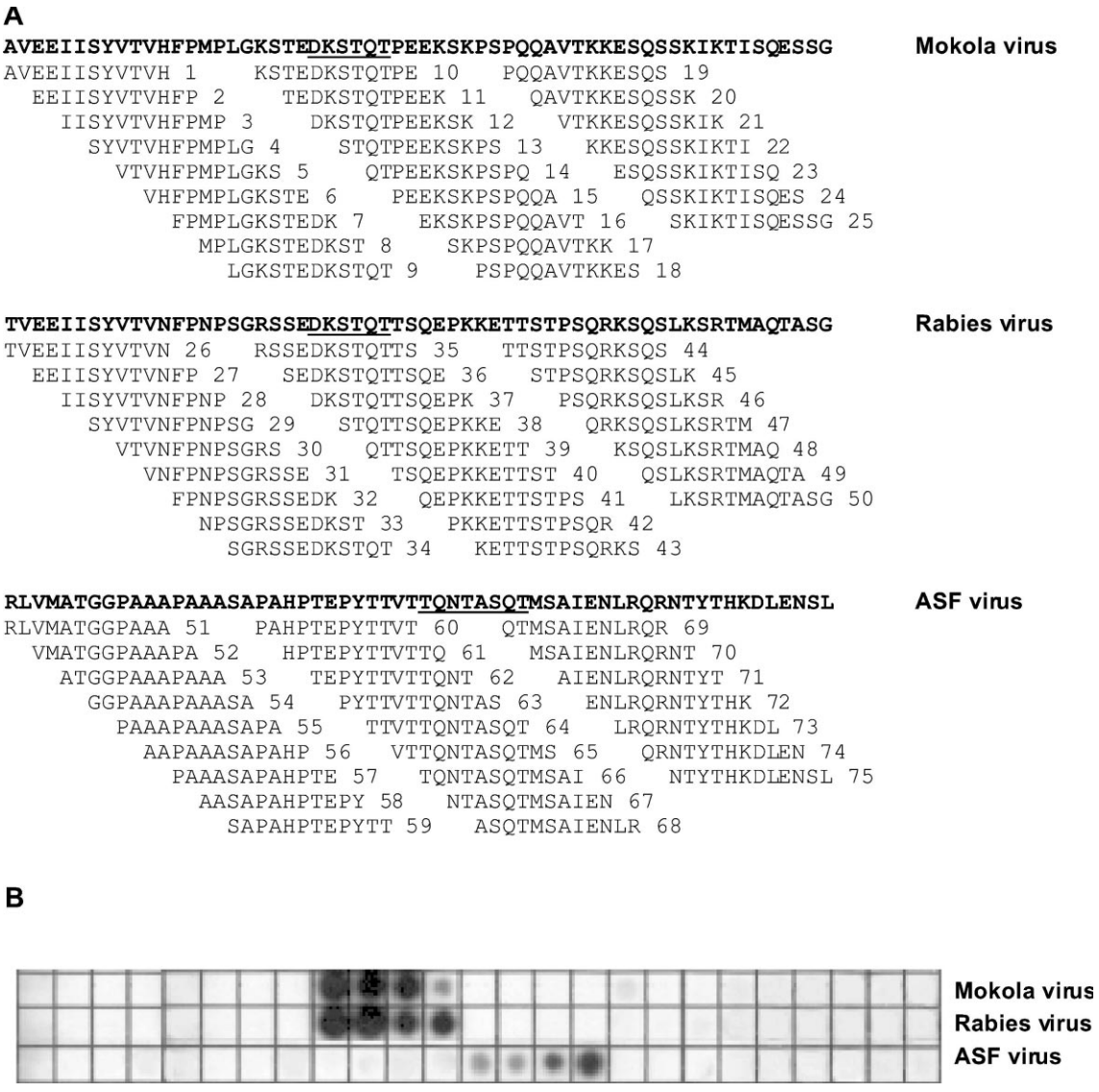

Fig. 3. Binding of LC8 to viral targets. Recombinant LC8 was incubated with a cellulose membrane bearing 75 spots with synthetic dodecapeptides that cover sequences corresponding to three different viral proteins (A). 25 dodecapeptides were synthesised on each line. The membrane was developed by ECL after incubation with a peroxidase-labelled anti-hexahistidine tag antibody (B). The underlined sequences represent the overlap region among consecutive positive spots.

subset of them become associated with the dynein multiprotein motor. On the other hand, both the NMR and crystal structures of LC8 bound to target peptides reveal that the flanking residues also establish binding interactions that stabilise the structure of the complex. Accordingly, we decided to inspect if the residues that appear just before and after this minimal binding sequence might somehow modulate the binding to LC8 and synthesised the KSTQT pentapeptide in different sequence environments, starting with the twenty natural amino acids (Fig. 4, left column). Although the LC8 recognition of the KSTQT motif is favoured by flanking residues such as Gln, Glu, Thr, Ser, Asp, Gly, His or Lys, it is virtually abolished by flanking residues such as Trp, Tyr, Ile, Pro, Cys, Val and Asn. Amino acids in the first group are either charged or polar (with the exception of Gly) while the amino acids in the second group are mostly apolar (with the exception of Asn). These pieces of data are in agreement with the flanking residues that appear in the ten proteins characterised previously that exhibit the KSTQT motif (Table 1). Next, we analysed the effect of the position of the KSTQT motif in the dodecapeptide by keeping a constant sequence (G, S or GS) at both ends. Since the dodecapeptides are synthesised starting from the C-terminus end, the N-terminus is likely to be more mobile, while the C-terminus end might be, in principle, less accessible for LC8 to bind. Surprisingly, the general binding trend seems to favour positions close to the C-terminus end of the dodecapeptide, with the last three positions being clearly preferred in any of the three groups of flanking residues (Fig. 4, right column). Strangely enough, when the KSTQT motif is positioned between residues 5 and 9, it displays a lower interaction with LC8 than at positions 4–8 or 6–11, probably due to unpredictable conformational effects of the synthetic peptides.

4. Discussion

Dyneins are highly complex, microtubule-based molecular motors that are involved in multiple motile events in the cy-

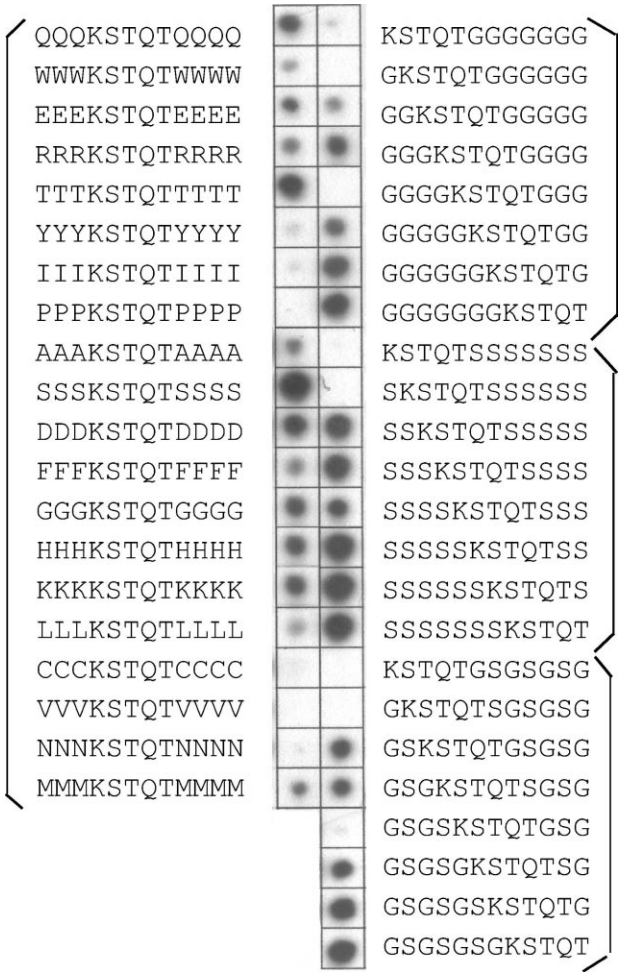

Fig. 4. Binding of LC8 to the KSTQT motif positioned in different sequence environments. Recombinant LC8 was incubated with a cellulose membrane bearing 44 spots with synthetic dodecapeptides where the KSTQT is introduced within different sequence environments. The membrane was developed by ECL after incubation with a peroxidase-labelled anti-hexahistidine tag antibody.

toplasm (e.g. maintenance of the Golgi apparatus, vesicular transport, spindle formation, etc.) and also function to provide the motive force for ciliary and flagellar beating [1–3]. The molecular mass of the dynein motor is estimated to be approximately 1–2 MDa, due to the numerous polypeptides that form the dynein molecular complex, including HCs, ICs, LICs and LCs. Two of the LCs located within the basal domain of the dynein particle, LC8 and Tctex1, shared by both flagellar and cytoplasmic dyneins, have also been recently regarded as tight binders of cargo.

The LC8 polypeptide was first identified as an integral component of the *Chlamydomonas* outer dynein arm where it is associated with the ICs at the base of the soluble particle [8]. Remarkably, subsequent biochemical studies have determined that the LC8 protein is a stoichiometric component of both brain cytoplasmic dynein and also the actin-based molecular motor, myosin V [5,13,19]. The three-dimensional structure of LC8 has been solved recently by both crystallography [15] and multi-dimensional NMR [20–22]. Following the isolation of LC8 as a nNOS interacting protein capable of dissociating the nNOS dimeric structure [9], two subsequent reports seemed to challenge this hypothesis suggesting that LC8 might be just involved in nNOS binding and its transport along microtubules towards the post-synaptic neuronal end [14,23]. Interestingly, in the last few years, at least nine additional proteins, apparently with no connection among them, have been reported to bind to LC8 using a yeast two hybrid approach [9–13,16–18].

We report herein a new method to inspect the interaction of both putative and previously described LC8 binding proteins with dynein LC by means of the pepscan technique. Synthesis of peptides composed of 12 consecutive amino acids bound to a solid support (cellulose membrane) allowed us to perform a fine mapping of the sequence requirements present in LC8 interacting proteins. Optimisation of the assay using nNOS as a control sequence confirmed the validity of our approach, since the binding was restricted to the sequence stretch present in the crystal and NMR structures [15,20–22]. Two homologous sequences to the nNOS motif KDTGIQVD were found in GKAP (LSIGIQVDD and also QSVGVQVEE) and in cytosine methyl transferase (KDLGIQVDR) (Table 1). Sequence comparison among the proteins that possess the KDTGIQVD motif, including two other nNOS sequences (human and rabbit), enables us to predict that it is very likely that human proteins such as centaurin β2 (with a DKDSGVRYDRV sequence), as well as KIAA0041 protein (with a MKDLGAQLDRD), will also be associated with LC8 in vivo.

Using the same methodology, we have been able to identify several novel proteins that also bind to LC8 through sequences that are identical or homologous to the KSTQT motif (Fig. 2). Kid-1 renal transcription factor is known to associate to LC8 in a yeast two hybrid experiment (Dr Ralph Wirzgall, personal communication). Our data indicate that this interaction is mediated by a TKSTQTQD motif (Fig. 2) present in its sequence downstream the Krüppel-associated box (KRAB) B [24]. In a similar manner, microtubule-associated protein 4 binds to LC8 through a SKSTQTVA polypeptide stretch and dynein HC through a VQDKLVQTPL motif. Finally, both Bim and the *Drosophila* swallow protein, two other proteins known to bind to LC8 in a yeast two hybrid methodology display the KSTQT and KATQT motifs respectively, and also interact strongly with LC8 according to our results.

When all the polypeptide sequences that successfully interact with LC8 were compared to each other, we could infer that although the KSTQT motif is highly conserved among LC8 interacting proteins, the Ser residue at the second position can be substituted by Ala, Leu or even Gln (Table 1). Support for this is provided by random mutagenesis studies of the LC8 binding sites of Bim [16] and glutathione *S*-transferase constructs [25], that also constrict the binding site to this pentapeptide.

Both NMR [20,21] and site-directed mutagenesis data [25] indicate that LC8 needs additional contacts with the side chain of residues flanking the KSTQT motif when interacting with a target protein. Positioning the KSTQT motif between the 20 possible natural amino acids enabled us to investigate the various side chains of amino acids that are capable of providing constructive interactions inside the LC8 binding groove (Fig. 4). Interestingly, the presence of the KSTQT stretch within a synthetic dodecapeptide is not sufficient to provide a positive interaction with LC8. However, polar residues such as Asp, Ser, Thr or Asn preceding the KSTQT motif convey on LC8 the ability to recognise the amino acid

pattern (Table 1, Fig. 4), unlike hydrophobic residues such as Tyr, Ile, Pro, Cys or Val, that abrogate this interaction (Fig. 4).

It is tempting to speculate that due to the dimeric structure of LC8 [14,15,26], one of the binding sites might be occupied by members of the dynein protein complex, with the other one being used for cargo transport. This inference is strengthened by the observed binding of LC8 to the dynein HC through the VQDKLVQTPL motif (Fig. 2) and by sequence alignment programs that revealed that dynein IC, known to be present with LC8 in a 1:1 stoichiometric ratio in cytoplasmic dynein [5], displays a YSKETQTPL motif in its sequence. In addition, an elegant report from Zhang and coworkers has recently demonstrated that dynein IC is also able to interact with LC8 [25].

It is also noteworthy, in this context, that two viruses from distant families share a common way of interaction with LC8 that most likely has an immediate importance in the viral life cycle. In both cases, this interaction results in the direct targeting towards the nuclear membrane, suggesting that viral proteins are able to hijack the cellular machinery in order to gain access to the nuclear membrane.

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