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Online Supplemental Material

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Supplemental material and methods

Antibodies

Further references concerning monoclonal antibodies used in this study can be found in the following articles: 6C4 (Kobayashi, T., E. Stang, K.S. Fang, P. de Moerloose, R.G. Parton, and J. Gruenberg. 1998. *Nature*. 392:193–197); CTR433 (Jasmin, B.J., J. Cartaud, M. Bornens, and J.P. Changeux. 1989. *Proc. Natl. Acad. Sci. USA*. 86:7218–7222); anti-HA (Daro, E., P. van der Sluijs, T. Galli, and I. Mellman. 1996. *Proc. Natl. Acad. Sci. USA*. 93:9559–9564); OKT9 (Sutherland, R., D. Delia, C. Schneider, R. Newman, J. Kemshead, and M. Greaves. 1981. *Proc. Natl. Acad. Sci. USA*. 78:4515–4519).

Cloning of CLIPR-59 and phylogenetic analysis

Human EST database was screened using the NCBI BLAST web server. Sequences showing detectable conservation with the CLIP-170 MTB motifs were then grouped in classes according to their similarity with already identified CLIP-related proteins (e.g., CLIP-170, CLIP-115, $p150^{glued}$, etc.). Three novel CLIP-170–related sequences were identified: EST-H1, -H4, and -H5 (EST-H1 was found to be the human homologue of cofactor-B; Tian, G., S.A. Lewis, B. Feierbach, T. Stearns, H. Rommelaere, C. Ampe, and N.J. Cowan. 1997. *J. Cell Biol.* 138:821–832). EST-H4 was initially characterized further by building up a contig of overlapping EST sequences. Two I.M.A.G.E. Consortium clones were obtained: No. 26245 (from nt 531 to 2278 of CLIPR-59) and No. 35186 (from 988 to the 3′ end). RACE was then performed on HeLa cDNA to complete the sequence in the 5′ direction, and the additional part of cDNA was fused to clone No. 26245. The resulting cDNA was used to generate the different tagged versions of full-length CLIPR-59 and deletion constructs. We also identified in the EST database a complete CLIPR-59 cDNA, EST29390, that we obtained from T.I.G.R. CLIPR-59 cDNAs were sequenced on both strands using the T7 polymerase dideoxy sequencing kit (Amersham Pharmacia Biotech). Alternatively, sequences were obtained by semi-automatic fluorescent sequencing (ABI). The genomic sequence of CLIPR-59 (GenBank/EMBL/DDBJ accession No. AC002116) allowed us to confirm our sequence and determine splicing sites. It should be noted that a strong conservation exists between the last 200 nt of CLIPR-59 cDNA and a rat thyrotropin releasing hormone sequence (GenBank/EMBL/DDBJ accession No. D17469) in the opposite orientation. Because this conservation was not observed with other rat thyrotropin releasing hormone sequences, this is likely due to a cloning artifact. Similar methods were used to identify the set of CLIP-170–related sequences in the fully sequenced organisms yeast, *Drosophila*, and *Caenorhabditis elegans*. The conserved MTB motifs of these proteins and other selected CLIP-170–related proteins were extracted from the full–length sequences and aligned by ClustalX (Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. *Nucleic Acids Res*. 24:4876–4882). The phylogenetic tree (neighbor joining) was generated from this alignment by ClustalX. Confidence in the groupings was estimated by bootstrap analysis with results as indicated on the tree (1,000 bootstrap trials). Groupings found in >90% of trials (indicated by a black circle) can be regarded as strongly supported, those found in >75% (gray circle) of trials as moderately supported, and those found in >50% of trials (white circle) as suggestive only. Members of the dynactin subfamily do exist in *C. elegans* and *Saccharomyces cerevisiae* (as judged by sequence homology outside of the MTB motif and/or other evidence), but these sequences grouped strongly with no other sequences. Indeed, different iterations of the analysis placed them at a low confidence level in a variety of positions.

Database analysis allowed us to assemble the sequence of mouse CLIPR-59 that shows a strong conservation with human CLIPR-59 (98% identity). This suggests a strong selection pressure that may be due to interaction with multiple partners. It is worth noting that stretches of sequences spanning the ankyrin domain and showing high sequence conservation with CLIPR-59 (70–80% identity) are present in the sequence databases of the fishes *Tetraodon nigroviridis* and *Ictalurus punctatus*. We, however, could not detect CLIPR-59 homologues in invertebrates or unicellular eukaryotes, even in organisms where the genome has been completely sequenced. More precisely, although other CLIP-170–related proteins are present in these organisms, none of them also contain ankyrin repeats. It will be important to understand whether these organisms do not actually have a CLIPR-59 homologue, or whether the role of the ankyrin domain can be fulfilled by another domain or a separate regulatory protein.

Plasmid construction

CLIPR-59 was tagged at its amino-terminal extremity or deleted in the amino-terminal region by double strand oligonucleotide insertion or by PCR mutagenesis to insert a NdeI site containing an in-frame ATG. Mutated inserts were then subcloned into a pBluescript II plasmid containing a KpnI/NdeI linker allowing in-frame fusion with a nonclassical HA tag (QDLPGNDNSTAGH; Daro, E., P. van der Sluijs, T. Galli, and I. Mellman. 1996. *Proc. Natl. Acad. Sci. USA*. 93:9559–9564). The carboxy-terminal deletion construct ΔC60 was made by adaptor insertion at the BamHI site of CLIPR-59. The CLIPR-59-C60 construct was obtained by joining the upstream NdeI site (from HA•CLIPR-59) to the internal BamHI site after Klenow filling in. GFP constructs were made by replacing the HA tag by an EGFP insert amplified by PCR to add an upstream KpnI site and a downstream, in-frame, AseI site. All the constructs were subcloned into the expression vector pCB6 under the CMV promoter to allow for expression in cultured mammalian cells after transfection by the calcium phosphate precipitate method (Jordan, M., A. Schallhorn, and F.M. Wurm. 1996. *Nucleic Acids Res*. 24:596–601), or in pSP72 under the T7 promoter for vaccinia virus–based overexpression system.