Online Data Supplement

Nuclear Import of Serum Response Factor in Airway Smooth Muscle

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Material and Methods

Plasmid construction:

Glutathione S-transferase-KPNB1 (GST-KPNB1) was a gift from Michael Malim. Human KPNA1 and KPNA2 in pRSET vectors (Invitrogen) and human KPNA3 in pBluescript (Stratagene) were gifts from Karsten Weis. PGEX-2T/KPNA4 was provided by Yoshihiro Yoneda; the human KPNA4 cDNA was excised and ligated into pBluescript (pBscr) to create pBscr/KPNA4. These plasmids were used as template for all derivative KPNA plasmids.

Primers incorporating a BamH1 site at the 5′ and Xho1 site at the 3′ end were used to generate KPNA1, KPNA2, and KPNA4 cDNAs, which were ligated into BamH1- and Xho1-digested pGEX KG (Amersham Pharmacia Biotech), to generate pGEX-KPNA1, -KPNA2, and -KPNA4. pGEX-KPNA3 was constructed in an analogous fashion, using PCR primers incorporating a HindIII site at the 5′ end and an Xho1 site at the 3′ end to generate the KPNA3 cDNA. The resultant plasmids were used for expression of GST-KPNA fusion proteins in E. coli. pGEX-SRF was provided by Mahesh Gupta, and was used for expression of GST-human SRF fusion protein in E. coli, and as a PCR template for derivative SRF cDNAs.

cDNAs encoding wild type (wt) KPNA1-4 were generated via PCR and ligated into the pCMV-Tag 5 mammalian expression vector (Stratagene), which appends a myc tag at the C-terminus; these plasmids are designated pKPNAx-myc (where x is 1-4). Primers, incorporating a 5′ BamH1 site and a 3′ Xho1 site were used to generate wt KPNA1, -2, and -4 cDNAs. KPNA3 (1) was constructed using primers incorporating a 5′ HindIII site and a 3′ Xho1 site. All 5′ primers incorporated a Kozac translation initiation sequence (GCCGCC) immediately upstream of the start codon. Additionally, the 3′ primers changed the stop codon in all constructs to glycine. To construct a cDNA encoding C-terminally hemaglutinin (HA)-tagged SRF (SRF-HA), we incorporated into the 5′ primer a BamH1 site and a Kozac sequence. The 3′ primer included an Xho1 site, changed the stop codon in SRF to glycine, and incorporated an HA tag sequence (reverse complement = ATGTACCCATACGATGTTCCAGA TTACGCT), followed by a stop codon (TTA). This PCR product was digested with BamH1 and Xho1 and ligated into similarly digested pcDNA3.1 (Invitrogen), to yield the mammalian expression plasmid pSRF-HA.

A series of mammalian expression plasmids encoding EGFP-SRF fusion proteins were constructed as follows. pGEX-SRF was digested with BamH1 and the wt SRF cDNA ligated into pEGFP-C1 (Clontech) to generate pEGFP-SRF. cDNAs encoding EGFP-mutant SRF fusion proteins were generated using PCR site-directed mutagenesis (GC-rich PCR kit [Roche]) as described (2). In mNLS-SRF, the SRF NLS was mutated by substituting glutamic acids for arginines at residues 95 and 96, as described by Gauthier-Rouviere (7). We generated a dimerization deficient SRF mutant, 5A-SRF, in which the hydrophobic span 183-VLLLV-187 in the dimerization domain (Figure 1) was replaced with 183-AAAAA-187. mNLS-5A-SRF contains both the NLS and 5A dimerization mutations. cDNAs encoding these SRF mutants were ligated in place of the wt SRF

cDNA in pEGFP-SRF, to generate expression plasmids encoding each EGFP-mutant SRF fusion protein. Pm1-SRF contains three point mutations (143-RVKI-146 → 143-LVAG-146) and inhibits DNA binding, as described by Johansen (3). The cDNA encoding pm1-SRF was excised from a pCGN vector (from Mahesh Gupta) by digestion with BamH1, and was ligated into similarly digested EYFP-C1 (Clontech), to yield pEYFP-pm1-SRF.

Bacterial expression plasmids were also constructed to generate SRF-EGFP fusion protein for use in a nuclear import assay (see below). A cDNA encoding wt SRF with the N-terminal tag LEVLFQGPGIRRASV was generated by PCR, and ligated into the BamH1 site in pGEX4TH-EGFP (gift from Stephen Adam). E. coli transformed with the resultant plasmid were used to express GST-(LEVLFQGPGIRRASV)-SRF-EGFP; the GST protein was bound to Glutathione Sepharose 4B beads (Pharmacia Biotech) then incubated with PreScission Protease (Amersham Biosciences), which cleaves the sequence LFQGP between glutamine and proline, thus releasing SRF-EGFP from GST. GST- (LEVLFQGP)-KPNA1 was expressed in bacteria from pGEX KG-KPNA1, and KPNA1 protein liberated from GST in a similar fashion. For all bacterially expressed proteins, the size and purity were assessed by SDS-PAGE electrophoresis and Coomassie staining.

Buffers:

Protein interaction buffer: 20 mM HEPES, 75 mM KCL, 1mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, 0.5% NP-40

Cytoskeleton buffer: 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM glucose, pH 6.1

Transport buffer: 50 mM Hepes, 110 mM KAc, 5 mM NaAc, 2 mM MgAc, 1 mM EGTA, 1 mM DTT, pH 7.3

ATP regenerating system: 30 mM creatine phosphate, 0.125 mg/ml creatine phosphokinase, 2 mM ATP, 2 mM GTP

Immunoprecipitation:

HEK293 cells were grown in DMEM/F12 media (Gibco/Invitrogen) containing 10% fetal calf serum, non-essential amino acids, and antibiotics (penicillin/streptomycin) and transiently transfected (as above) with pSRF-HA as well as pKPNA1-4-myc, or corresponding empty vectors as negative control. Cytoplasmic (CE) and nuclear (NE) extracts were prepared using standard reagents and protocols (Pierce). Protein concentrations were measured by Bradford assay to ensure equivalent conditions for IP experiments. CE and NE were pre-cleared with normal mouse IgG agarose-conjugated (AC) beads (Santa Cruz) on ice for 30 min. Cleared supernatants were incubated with AC beads loaded with mouse monoclonal anti-HA antibodies (Santa Cruz). Tubes were rotated for 1.5 hrs at 4°C, then beads were pelleted, washed in cold PBS, resuspended in Laemlli's sample buffer and boiled for 5 min. Beads were spun down and the supernatants (containing immunoprecipitated proteins) were analyzed by SDS-PAGE and western blotting with rabbit polyclonal anti-c-myc antibody (Santa Cruz), horseradish peroxidase-linked donkey anti-rabbit secondary antibody (Amersham), and chemiluminescent detection (Pierce). Uncleared CE or NE were also included on western blots to confirm KPNA expression.

Figures

Figure 1. Serum response factor (SRF). (a) Functional regions of SRF. (b) Amino acid (aa) sequence of DNA binding region in SRF. AA involved in SRF dimer formation are highlighted [as determined by three-dimensional crystal structure of SRF bound to DNA.] AA mutated in this study are underlined. (c) SRF constructs utilized in this study.

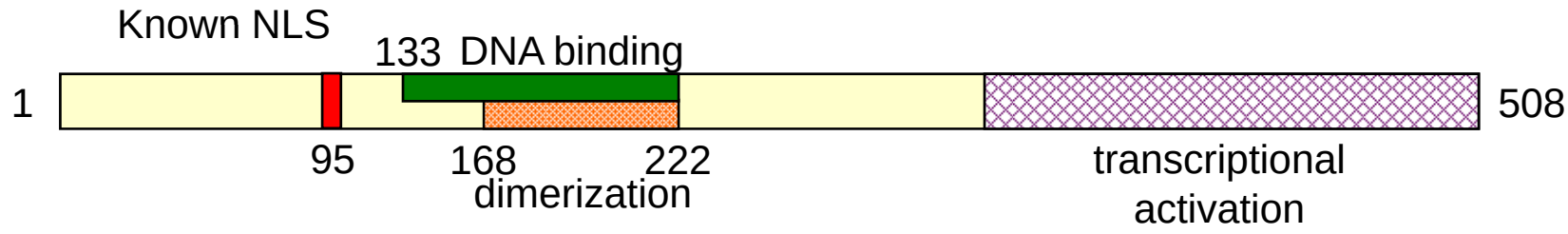
Figure 2. Potential mechanisms of SRF nuclear import. (A) SRF binds to KPNA1 which in-turn binds to KNPB1 to facilitate nuclear entry. (B) SRF binds directly to KPNB1 to gain nuclear entry. (C) SRF gains nuclear entry by an NLS-independent mechanism, as depicted by mNLS-SRF.

References

1. XM_165637. Homo sapiens karyopherin alpha 3 (importin alpha 4) (kpna3), mrna.
2. Fu Y, Liu HW, Forsythe SM, Kogut P, McConville JF, Halayko AJ, Camoretti-Mercado B, Solway J. Mutagenesis analysis of human sm22: Characterization of actin binding. *J Appl Physiol* 2000.
3. Johansen FE, Prywes R. Identification of transcriptional activation and inhibitory domains in serum response factor (srf) by using gal4-srf constructs. *Mol Cell Biol* 1993;13(8):4640-4647.

SERUM RESPONSE FACTOR

A



B

133 GAKPGKKTRGRVKIKMEFIDNKLRRYTTFSKRKTGIMKKAYELST 177

178 LTGTQVLLLVASETGHVYTFATRKLQPMITSETGKALIQTCLNSP 222

C

wt	95 RRGLKR 100 and 183 VLLLV 187
mNLS	95 RRGLKR 100 → 95 EEGLKR 100
dimer mutant (5A)	183 VLLLV 187 → 183 AAAAA 187
pm1	143 RVKI 146 → 143 LVAG 146

Figure 1

SRF: Potential nuclear import mechanisms

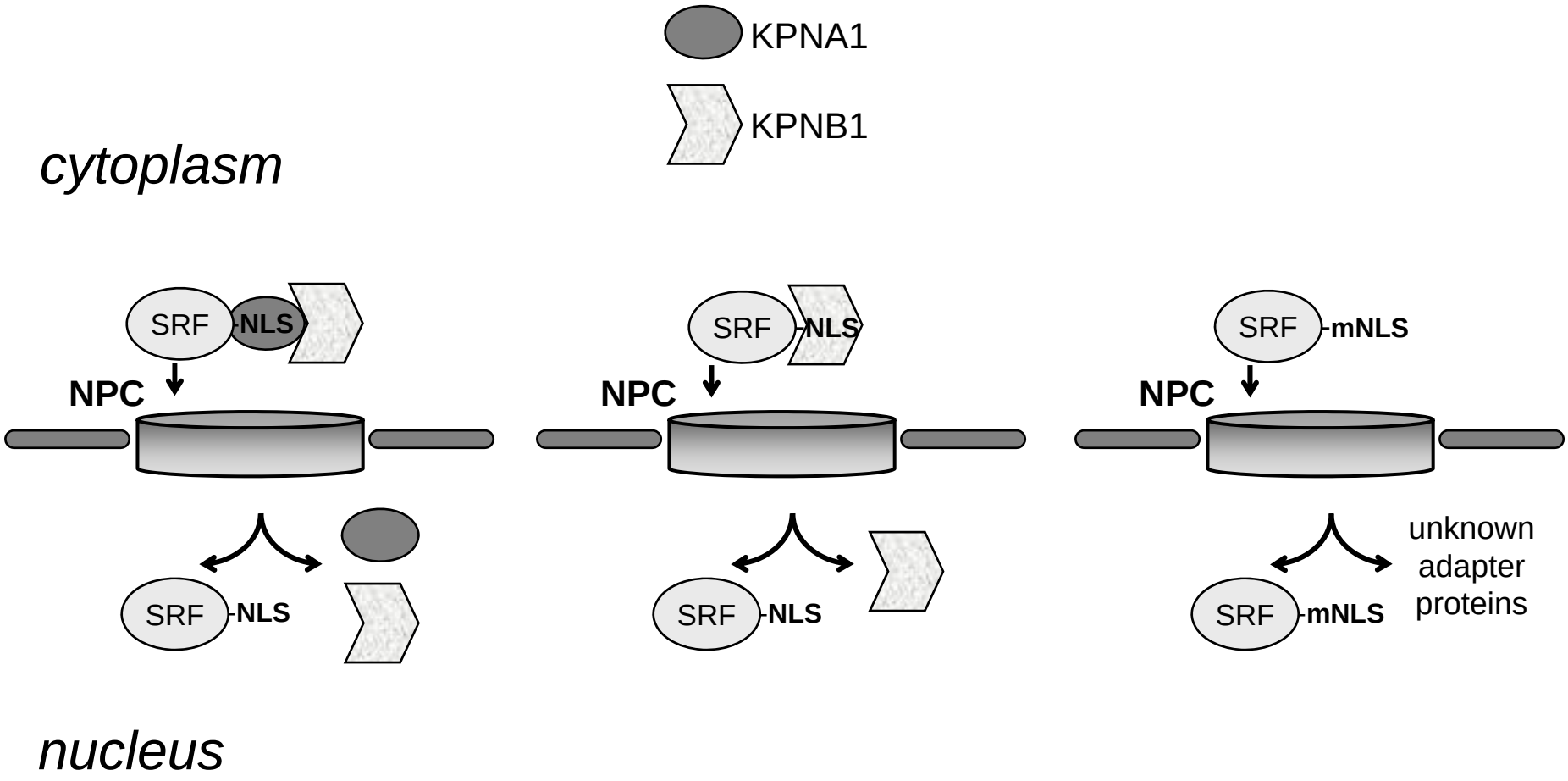


Figure 2