The PSF•p54nrb Complex Is a Novel Mnk Substrate That Binds the mRNA for Tumor Necrosis Factor α*

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To identify new potential substrates for the MAP kinase signal-integrating kinases (Mnks), we employed a proteomic approach. The Mnks are targeted to the translational machinery through their interaction with the cap-binding initiation factor complex. We tested whether proteins retained on cap resin were substrates for the Mnks in vitro, and identified one such protein as PSF (the PTB (polypyrimidine tract-binding protein)-associated splicing factor). Mnks phosphorylate PSF at two sites in vitro, and our data show that PSF is an Mnk substrate in vivo. We also demonstrate that PSF, together with its partner, p54nrb, binds RNAs that contain AU-rich elements (AREs), such as those for proinflammatory cytokines (e.g. tumor necrosis factor α (TNFα)). Indeed, PSF associates specifically with the TNFα mRNA in living cells. PSF is phosphorylated at two sites by the Mnks. Our data show that Mnk-mediated phosphorylation increases the binding of PSF to the TNFα mRNA in living cells. These findings identify a novel Mnk substrate. They also suggest that the Mnk-catalyzed phosphorylation of PSF may regulate the fate of specific mRNAs by modulating their binding to PSF•p54nrb. DNA relaxation, and tumorigenesis (reviewed in Ref. 1). These proteins also cooperate in the inhibition of human immunodeficiency virus type 1 mRNA expression (2). Moreover, PSF is also been reported to repress gene expression through its association with nuclear hormone receptors (3) or through binding to insulin-like growth factor-1 response elements (4). PSF and p54nrb are both phosphoproteins. Phosphorylation may be involved in the relocalization of PSF during apoptosis (5) and in regulating the binding properties of p54nrb during mitosis (6).

All eukaryotic cytoplasmic mRNAs have a 5′-terminal cap structure that contains 7-methyl-GTP (m7GTP) and promotes their efficient translation (7, 8). The cap is bound by eukaryotic translation initiation factor eIF4E, which also binds to the scaffold eIF4G and through this with other translational factors to recruit the 40 S ribosomal subunit to the mRNA. eIF4G also binds the poly(A)-binding protein (PABP), which interacts with the 3′-end of the mRNA, thus circularizing it (reviewed in Ref. 9).

eIF4E is phosphorylated in vitro and in vivo by the MAP kinase-signal integrating (or MAP kinase-interacting) kinases (Mnks) (10–13). There are two Mnk genes in humans, each of which generates two different polyptides as a consequence of alternative splicing (14, 15). The longer Mnk1 isoform, Mnk1α, is switched on by signaling through the ERK and p38 MAP kinase pathways, whereas Mnk2a (the longer Mnk2 isoform), in contrast, shows high basal activity (11, 16, 17). We recently showed that Mnks play an important role in the control of the production of the proinflammatory cytokine tumor necrosis factor α (TNFα) in T-cells (18).

The 3′-untranslated regions of the TNFα mRNA and many other cytokines contain regulatory AU-rich elements (AREs), which regulate their stability and/or translation through their interaction with ARE-binding proteins (19). Specific stimuli increase the stability and/or translation of such mRNAs, and this is important in controlling the production of cytokines such as TNFα (reviewed Ref. 20), which is regulated through signaling pathways that include ERK and p38 MAP kinase α/β (21), i.e. the pathways that regulate Mnk1. Here, we show that PSF•p54nrb interacts with ARE-containing RNAs and that PSF is phosphorylated and regulated by the Mnks.

MATERIALS AND METHODS

Chemicals—All chemicals and biochemicals were from Sigma.

Antibodies—These were obtained as follows: anti-human eIF4E was raised against a synthetic peptide corresponding to

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residues 5–23 of the protein, anti-PSF phospho-Ser\textsuperscript{8} was raised against a synthetic peptide corresponding to residues 2–13 of the protein (at Diagnostics Scotland), anti-eIF4E and anti-PARP were kindly provided by Dr. Simon Morley (University of Sussex). Anti-human CD3 was a kind gift from Dr. R. Vilella (Hospital Clinic, Barcelona) and was used at 1:1000 dilution. Anti-human CD28 was a kind gift from Dr. Pedro Romero (Ludwig Institute for Cancer Research, Switzerland) and was used as a hybridoma supernatant at 1:20 dilution. Other antibodies used were anti-p54\textsuperscript{rb} (BD Transduction Lab), anti-PSF (monoclonal antibody, clone B92, Sigma), anti-TIA-1 (C-20, Santa Cruz Biotechnologies), anti-hemagglutinin (monoclonal antibody, Roche Diagnostics), anti-myc and anti-FLAG monocl- onal antibodies (Sigma), anti-α-tubulin and anti-lamin B (Santa Cruz Biotechnolog-

Affinity Resins—7-Methyl-GTP-Sepharose 4B was from Amersham Biosciences, Sepharose CL-4B (control for nonspecific binding to Sepharose beads) was from Sigma, nickel-nitrilotriacetic acid agarose was from Qiagen, and streptavidin-agarose from Invitrogen.

Plasmids—pET15b-PSF was a kind gift from Dr. James Patton (Vanderbilt University), pET15b-p54\textsuperscript{rb} was obtained from pEYFP-p54\textsuperscript{rb} (BD Transduction Lab), anti-PSF (monoclonal antibody, clone B92, Sigma), anti-TIA-1 (C-20, Santa Cruz Biotechnologies), anti-hemagglutinin (monoclonal antibody, Roche Diagnostics), anti-myc and anti-FLAG monocl-

Protein Expression and Purification—Recombinant eIF4E was obtained as described previously (22). Recombinant PSF and p54\textsuperscript{rb} were expressed as His-tagged proteins in Esche-

RNA Affinity Chromatography—RNA affinity chromatography was performed as described (23) with biotinylated RNA oligoribonucleotides encoding the AU-rich region of mouse TNF\textalpha (UUAAUUAAUUAUUUAUUAAUUAAUUAAUU), mouse granulocyte-macrophage colony stimulating factor (UUAAUUAAUUAUUAAUUAAUUAAUUAAUU), human c-Fos (GUUUAUAUUAUUAAUUAAAG-AUGGAUUC), mouse interferon γ (AAACCUUAUUAAUAAUUAAAAACUUUAUAAUAG), human COX-2 (CUA-AUGAUAUAUAUUAAUUAAAUAAUGAACCAUA), or a negative-control sequence (AAGCUCUGCGCCAGGGCAGCG-AUCUCAGAGGA). The TNF\alpha and negative-control oligo RNAs were purchased from Dharmacon, the rest were a kind gift from Dr. Simon Rousseau (University of Dundee, Scotland).

In Vitro Binding Assays—In vitro RNA-binding assays were performed in lysis buffer containing 20 mM HEPES (pH 7.4), 50 mM KCl, 50 mM β-glycerophosphate, 0.2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 mM benzamidine, and 1 mM dithiothreitol supplemented with 5 μg/μl bovine serum albumin. The specified protein(s) (PSF, p54\textsuperscript{rb}, and/or eIF4E, 30 pmol) and, where indicated, 175 pmol of biotinylated oligoribonucleotide containing the TNF\alpha AREs, were incubated for 1 h at 4 °C prior to the addition of the resin (m7GTP-Sepharose, Sepharose CL-4B, nickel-agarose, or streptavidin-agarose beads, 15 μl of packed beads). The mixture was then incubated for a further 1 h at 4 °C. Then the beads were washed 3 times with lysis buffer and resuspended in SDS sample buffer to be analyzed by SDS-PAGE and immunoblotting.

Resin Chromatography—After washing with PBS, 1 × 10\textsuperscript{14} plate of human embryonic kidney (HEK) 293 cells were lysed with 400 μl of 20 mM HEPES (pH 7.4), 50 mM KCl, 50 mM β-glycerophosphate, 0.2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 mM benzamidine, and 1 mM dithiothreitol. The lysates were centrifuged for 5 min at 10,000 × g to remove cell debris. The supernatant was incubated with 20 μl of packed beads of m7GTP-Sepharose or Sepharose CL-4B. After 2 h the beads were washed 3 times with lysis buffer. Unless stated otherwise, beads were resuspended in SDS sample buffer to be analyzed by SDS-PAGE and immunoblotting. The material retained on m7GTP-Sepharose was incubated with 0.1 mg/ml RNase A and 160 units/ml RNase T1 at 16 °C for various times (see legends).

Immunoprecipitations—2 μg of anti-myc, 6 μg of anti-PSF, or 15 μg of anti-FLAG antibodies were added to 300 μg of cell extracts, and the mixture was incubated for 1 h at 4 °C. Subsequently, protein G-Sepharose (20 μl of packed beads per immuno-

Ribonucleoprotein Immunoprecipitation Assay—Reversible cross-linking combined with IP to study RNA-protein interactions in vivo was performed as described previously (24). Briefly, 30 × 10\textsuperscript{6} Jurkat cells left untreated or activated with anti-CD3/anti-CD28 for 1.5 h were incubated with phosphate-buffered saline containing 1% formaldehyde. Sonicated lysates were subjected to IP with 2 μg of anti-PSF antibody. After extensive washing, the beads were incubated at 70 °C for 45 min to reverse the cross-links, the RNA was purified using TRIzol\textsuperscript{®} (according to the manufacturer’s instructions, Invitrogen), and the immunoprecipitated RNA was analyzed by RT-PCR or RT-

RT-PCR and RT-Q-PCR Amplification Analysis—Total RNA extracted using the RNeasy kit (Qiagen) or RNA extracted from the PSF immunoprecipitates (TRIzol method, Invitrogen) were treated with 160 units/ml RNase-free DNase I and then subjected to reverse transcription using SuperScript III (Invitrogen) and oligo(dT)$_{15}$/Random primers (Invitrogen) following the manufacturer’s instructions. Subsequently, PCR using the Platinum Taq DNA Polymerase (Invitrogen) was performed.
using specific primers to amplify cDNAs for either glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or TNFa. The primers used here are TCGGATCGAGGATTGGTGC and AGCAAGGATGATGTTCTGAGAG for GAPDH, and CAGAGGGAAGATTCCCGAG and CTTGGTCTCGG-TAGGAG for TNFa.

For RT-Q-PCR analysis, TaqMan Gene expression assays (Applied Biosystems) were used. References are Hs00174128_m1 for human TNFα, Hs99999901_s1 for the 18 S ribosomal RNA, and Hs00174086_m1 for interleukin-10.

**SDS-PAGE and Immunoblotting**—Cell lysates were prepared in Laemmlı sample buffer and heated for 5 min at 95 °C. Polyacrylamide gels ranging from 10 to 15% were used to analyze protein content of the lysates. The proteins were either transferred onto polyvinylidene difluoride membranes and detected by Western blot analysis or were transferred onto nitrocellulose membranes and detected by a fluorescence-based detection method (using the LiCOR Odyssey system).

**Cell Culture and Transfection Experiments**—Jurkat T-cells were grown in a 5% CO2 incubator at 37 °C in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) serum, penicillin, and streptomycin and cultured in 10-cm dishes in a 5% CO2 incubator at 37 °C. HEK293 cells were transfected using the calcium phosphate method whereby a calcium/phosphate (125 mM CaCl2, 50 mM BES, 280 mM NaCl, 1.5 mM sodium hydrogen orthophosphate)/DNA co-precipitate is added to the cells. HEK293 cells were split to a density of 2 × 106 cells per 10-cm diameter dish and after 8 h at 37 °C in complete Dulbecco's modified Eagle's medium, cells were transfected with 1 μg/ml DNA co-precipitated per dish. After transfection, cells were placed in the incubator at 37 °C and 5% CO2. If cells were to be stimulated, 24 h after transfection they were starved of serum overnight and then stimulated. Otherwise they were lysed 36 h after transfection. Where non-transfected cells were used, they were starved of serum overnight prior to stimulation, and then stimulated with 100 nm insulin, 1 μm tetracanoylphorbol 13-acetate (TPA), or 100 μm arsenite for 30 min.

**In Vitro Protein Kinase Assays and Peptide Mapping**—Recombinant His-tagged Mnk1 or -2 (0.04 µg/µl) was activated in vitro with active SAPK2α (p38 MAPKα, final concentration, 1 unit/ml; from DSSG, University of Dundee) and 100 µM ATP in kinase buffer (100 mM HEPES-KOH, pH 7.4, 250 mM KCl, 50 mM MgCl2, 25% glycerol, 1 mM dithiothreitol, and 0.5 mM Na2VO4). After 2 h at 30 °C, SB203580 at a final concentration of 10 μM was added. 5 μl of active Mnk was used to phosphorylate proteins bound to 20 μl of m7GTP-Sepharose in vitro in the presence of 100 μM ATP and [γ-32P]ATP. The reaction was stopped by adding Laemmli buffer with 10 mM dithiothreitol and heating for 5 min at 95 °C. Phosphorylated proteins were separated in a 15% acrylamide/bisacrylamide gel. Labeled bands detected by autoradiography were excised and subjected to tryptic digestion (25). Phosphopeptides were detected by precursor ion scanning in the negative ion mode as described previously (26).

**Active Mnk1 or -2 was also used to phosphorylate recombinant PSF.** The reaction was stopped by adding Laemmli denaturing buffer containing 10 mM dithiothreitol and heating for 5 min at 95 °C. Cysteines were alkylated with iodoacetamide for 45 min at 30 °C and proteins separated by SDS-PAGE. The labeled protein was excised from the gel and digested overnight with trypsin. Samples were acidified by adding 3 volumes of 0.1% trifluoroacetic acid in acetonitrile and peptides were separated by reverse-phase chromatography on a Vydac C18 (250 × 4.6 mm inner diameter) column equilibrated in 0.1% trifluoroacetic acid and developed with a gradient of acetonitrile. Fractions containing radioactivity were collected and analyzed by mass spectrometry (MALDI-TOF) and solid-phase sequencing. Two-dimensional mapping was described previously (27) using a second dimension buffer containing isobutyric acid.

**Cellular Fractionation**—10 × 106 Jurkat cells were harvested in 300 μl of EZ lysis buffer (Sigma) with the addition of 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml each of leupeptin, antipain, and pepstatin. After centrifugation for 5 min at 1,000 × g at 4 °C, the cytoplasmic fraction was trans-
ferred to a new tube. The nuclear pellet was washed twice with 500 μl of EZ Lysis buffer and then lysed by addition of SDS-PAGE sample buffer.

RESULTS

Mnks Phosphorylate PSF in Vitro—Because the Mnks bind to the eIF4F cap-binding complex and phosphorylate at least two of its components, eIF4E and eIF4G (10, 28), we reasoned that additional proteins associated with this complex might be substrates for the Mnks. We therefore performed affinity purifications from cytoplasmic lysates of HEK293 cells using m7GTP-Sepharose beads and incubated the bound material with highly active Mnk1 and [γ-32P]ATP (i.e., using a modified version of the KESTREL procedure (29)). The reaction products were analyzed by SDS-PAGE and autoradiography. Four major radiolabeled

FIGURE 2. PSF-p54 indic does not bind directly to eIF4E, 4E-HP, or CBP20. A, 30 pmol of His-PSF, His-p54 indic, and/or untagged recombinant eIF4E (made in bacteria) were used in in vitro binding assays with nickel-agarose beads (SN, supernatant). B, Western blots from anti-PSF or anti-FLAG immunoprecipitates obtained from HEK293 cells transfected with pcDNA3-CBP20 (hemagglutinin-tagged CBP20) or empty vector (Vector) and immunoprecipitates prepared from lysates from serum-fed cells, using anti-hemagglutinin.

FIGURE 3. Characterization of the retention of PSF-p54 indic on m7GTP-Sepharose. A, 30 pmol of recombinant His-PSF or His-p54 indic made in E. coli were assayed in vitro for binding to m7GTP-Sepharose or Sepharose CL-4B. B, HEK293 cell extracts (300 μg of protein) were supplemented with GTP or m7GTP at 0.5 or 1 μM and then subjected to m7GTP-Sepharose pull-down. The presence of PSF-p54 indic and eIF4E on the resin was analyzed by Western blotting. C, Western blots from RNAse-treated (0.1 mg/ml RNAse A and 160 units/ml RNAse T1 at 16 °C for the times indicated) and control (mock) m7GTP-Sepharose pull downs from insulin-stimulated HEK293 cells. The signal for eIF4E (the component that binds directly to the resin) serves as the "loading control" for interpreting the data for the other antibodies.
bands were observed (Fig. 1A). The lowest molecular weight band, as judged from its electrophoretic mobility corresponded to eIF4E. A prominent band corresponded to the added Mnk (see kinase-only (MnkI lane in Fig. 1A), which undergoes extensive autophosphorylation (Mnk was also identified by mass spectrometry, Table 1).

Two other major radiolabeled bands were excised from the gel. The proteins contained in these bands were identified by tryptic mass fingerprint analysis as elf4G and PSF (Fig. 1A, Table 1). Although elf4G and elf4E are known to be retained on m7GTP-Sepharose and be substrates for the Munks, PSF has not previously been reported to be retained on the cap resin or to be an Mnk substrate. The identification of PSF was confirmed by Western blot analysis (Fig. 1B). Also present among the proteins that bound m7GTP-Sepharose was p54\textsuperscript{nrb}, which associates with PSF (1) (Fig. 1B), as well as PABP, which interacts with elf4G (30). In contrast, the RNA-binding protein La was not found in the PSF band (Fig. 1B). Western blots for PSF (Fig. 2A) and elf4G (Fig. 2B) were stained with a polyclonal antibody to PSF (rabbit) and commercial anti-elf4G antibody, respectively.

**FIGURE 4. PSF-p54\textsuperscript{nrb} bind to ARE-containing RNAs.** A, Western blot from pull-downs from Jurkat T-cells using TNF\textalpha- or control oligoribonucleotide. B, Western blot from pull-downs with biotinylated oligoribonucleotides containing the AREs from the TNF\textalpha, granulocyte-macrophage colony stimulating factor (GM-CSF), c-Fos, interferon \gamma, and cyclooxygenase (COX) 2 mRNAs, or a negative control (Con). C, \textit{in vitro} binding of recombinant (Rec) His\textsubscript{6}-PSF and/or His\textsubscript{6}-p54\textsuperscript{nrb} to streptavidin-agarose beads in the presence or absence of biotinylated TNF\textalpha oligonucleotide (TNF oligo). Western blots for PSF (left) and p54\textsuperscript{nrb} (right) in the bound material (StAg) or supernatants (SN) are shown. D, TNF\textalpha and GAPDH mRNA levels in anti-PSF IPs or total cell lysates from Jurkat cells, determined by RT-PCR. E, the amounts of GM-CSF mRNA co-immunoprecipitating with PSF were assessed by quantitative PCR following reverse transcription of the RNA. The data are averaged for duplicates performed, similar data being obtained in three independent experiments, and are shown relative to the signal for the “no Ab” negative control.

To a known partner, elf4E-binding protein 1, demonstrating the utility of the approach (data not shown).

As a further test, elf4E was expressed in HEK293 cells as a myc-tagged fusion, and immunoprecipitated with anti-myc. No PSF or p54\textsuperscript{nrb} was recovered in the immunoprecipitate, whereas elf4G (a positive control that binds directly to elf4E) was recovered in this fraction (Fig. 2B). These findings again indicate the absence of a direct interaction of PSF-p54\textsuperscript{nrb} with elf4E or, probably, with elf4G.

To test for possible interactions with two other m7GTP-binding proteins, 4E-HP and CBP20, these proteins were individually expressed as FLAG- or hemagglutinin-tagged fusions in HEK293 cells, and then immunoprecipitated from the lysates. This did not result in the co-precipitation of PSF or p54\textsuperscript{nrb}, indicating that neither of these cap-binding proteins interacts with PSF-p54\textsuperscript{nrb} either directly or indirectly (Fig. 2, C and D).

To test whether PSF or p54\textsuperscript{nrb} could bind directly to m7GTP, purified recombinant PSF and p54\textsuperscript{nrb} (made in bacteria, which lacks cap-binding proteins) were mixed with m7GTP-Sepharose or, as a negative control, CL-4B beads. Very little retention of either protein on the beads was seen (Fig. 3A). Nevertheless, it remained possible that PSF-p54\textsuperscript{nrb} did interact directly with
PSF-p54nr, an Mnk Substrate That Binds TNF mRNA

the m7GTP-Sepharose beads, although because PSF and p54nr are both nucleotide (RNA)-binding proteins, the interaction could be nonspecific. We therefore studied the specificity of the binding of PSF-p54nr to this resin. To do this, we assessed the ability of PSF-p54nr, and as a control for a genuine m7GTP-binding protein, eIF4E, to bind m7GTP-Sepharose in the presence of an excess of free m7GTP or GTP nucleotides. As shown in Fig. 3B, eIF4E was not retained on the resin in the presence of m7GTP (as expected), whereas PSF-p54nr was excluded by either m7GTP or GTP. These data suggest that PSF or p54nr (or other components of nucleoprotein complexes in which they are involved) can bind to nucleotide affinity resins.

To test whether PSF-p54nr associated with the cap resin via binding to RNAs that are pulled down with this resin, we tested whether its recovery on m7GTP-Sepharose was affected by pretreatment of the bound material with RNase. As shown in Fig. 3C, treatment with ribonucleases A plus T1, but not “mock” incubation in the absence of nucleases, greatly decreased the amount of PSF and p54nr that was recovered in this way. Incubation had a small effect on the binding of eIF4G (which was the same for incubation with or without RNase) but essentially no effect on the amount of eIF4E recovered except at the final time point of the mock incubation where a modest decrease in all proteins was observed. Thus, it appears that the appearance of PSF-p54nr in the cap resin-bound material is due to their association with RNAs that are present in this material. A key question was therefore: do PSF-p54nr bind nonspecifically to mRNAs or are they preferentially associated with specific messages?

**PSF-p54nr Bind ARE-containing RNAs—**PSF was found during a study of proteins that bind to an oligoribonucleotide corresponding to the AU-rich element of the TNFα mRNA. To confirm this, we used a biotinylated oligoribonucleotide containing the AREs of the TNFα mRNA and, as negative control, one lacking AREs. PSF and p54nr were retained on the ARE-containing oligoribonucleotide but not the negative control (Fig. 4A). A negative control protein, eIF4G, was not retained on either oligonucleotide (Fig. 4A). This is to be expected, as whereas it binds mRNAs via its partners eIF4E and PABP, it should not bind these oligonucleotides as they are neither capped nor polyadenylated.

PSF and p54nr were retained on oligonucleotides corresponding to the ARE-containing regions of the TNFα, granulocyte-macrophage colony stimulating factor, c-Fos, interferon γ, and cyclooxygenase 2 mRNAs (Fig. 4B). Binding to the TNFα and interferon γ-based oligoribonucleotides was greatest. A well known ARE-binding protein, TIA-1, also bound to all these RNAs (Fig. 4B). Neither TIA-1 nor PSF or p54nr bound to the negative control RNA. To study this further, we tested whether recombinant (bacterially expressed) PSF or p54nr, alone or in combination, could bind to the biotinylated TNF ARE-based oligonucleotide in vitro. When tested alone, little retention of

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4 S. Rousseau, personal communication.
either protein on the RNA was observed (Fig. 4C). Only when they were added together was substantial binding observed. The ability to interact with ARE-containing RNA is thus a feature of the PSF-p54nrb complex rather than of either protein alone, and is a direct interaction, rather than being mediated via another mammalian oligopyrimidine tract-binding protein such as PTB (with which PSF associates (35)).

It was clearly important to establish whether PSF binds to the TNFα mRNA in vivo. We therefore performed the following IP RT-PCR experiment, using Jurkat T-cells that had been stimulated with antibodies to CD3 and CD28 rather than HEK293 cells because the latter do not produce TNFα. Activated Jurkat T cells, as well as unstimulated controls, were cross-linked using formaldehyde. Cell lysates were prepared and PSF was immunoprecipitated from them. RNA was extracted from the precipitates and TNFα mRNA detected by reverse transcription followed by PCR, using primers in different exons to ensure that we were looking at mature mRNA. As expected from earlier work (e.g. (18)), stimulation of Jurkat cells greatly increased the level of TNFα mRNA relative to the GAPDH housekeeping control (assessed by RT-PCR of whole cell lysates, Fig. 4D). In the PSF IP, a strong signal was seen with the TNFα PCR primers for stimulated cells, whereas no product was observed for non-stimulated cells, indicating that PSF binds the TNFα mRNA in vivo. No product was amplified from the anti-PSF pull-down when GAPDH primers were used (Fig. 4D). This demonstrates for the first time that PSF-p54nrb bind to a specific ARE-containing mRNA in living cells.

Because PSF also interacted in vitro with oligonucleotides based on the AREs from other mRNAs (Fig. 4B), we also attempted to perform similar IP RT-PCR studies for additional mRNAs. As shown in Fig. 4E, PSF was found to bind to the granulocyte-macrophage colony stimulating factor mRNA in lysates from stimulated Jurkat cells. However, in experiments not shown here, no signal was obtained using PCR primers for the interferon γ and interleukin-10 mRNAs. Further analysis revealed that the levels of these mRNAs are very low in Jurkat cells stimulated under the conditions used here. Further work is thus required to establish which mRNAs do bind specifically to PSF.

PSF Is Phosphorylated at Two Sites by the Mnks—It was important to confirm that PSF is indeed a Mnk substrate, as suggested by the data in Fig. 1A. To do this, we expressed PSF in E. coli as a hexahistidine-tagged fusion and incubated it with activated Mnk1 or Mnk2 (Fig. 5A). The Mnks clearly phosphorylate recombinant PSF in vitro (here, eIF4E serves as the positive control). To identify the phosphorylation sites, radiolabeled PSF was subjected to tryptic digestion and the resulting peptides were analyzed by reverse-phase HPLC (Fig. 5B) and two-dimensional mapping (Fig. 5C). Subsequent analysis by mass spectrometry and Edman degradation revealed that peak “b” from the reverse-phase HPLC corresponded to a peptide in which Ser8 was phosphorylated, peak “d” to a species containing Ser(I)283, and peak “e” to phosphorylated serines in the thrombin cleavage site of the recombinant fusion protein.

As confirmation of the identification of the sites, the S8A and S283A mutants of PSF were prepared and incubated with Mnk2 in vitro. The radiolabeled proteins were then subjected to tryptic digestion and peptide mapping (Fig. 5D). In the maps from the S8A mutant, peptide b and the weaker spots termed “a” and “c” were all absent. They all likely contain Ser8, being generated by alternative tryptic cleavage as this region contains multiple arginyl residues. Peptide d was absent in the map from the S283A mutant. Mnk-mediated phosphorylation of PSF thus occurs at Ser8 and Ser283. In fact, whereas Ser283 is phosphorylated similarly by Mnk1 and Mnk2, Mnk2 phosphorolyses Ser8 much better than Mnk1 does (Fig. 5E). This is not a reflection of differences in the relative activities of the two enzymes used in this assay, because the labeling of peptide d is at least as strong in the Mnk1-derived map, whereas peptides a–c are much weaker. Thus the relative levels of the labeling of the different sites achieved with Mnk1 and Mnk2 differ, and these data thus indicate a difference in specificity between Mnk1 and Mnk2. One peptide (“f” in Fig. 5E, upper section) is only seen in maps from PSF phosphorylated by Mnk1: as it is clearly a minor species, we have not attempted to identify it.

We then used synthetic phosphopeptides to prepare antisera for PSF phosphorylated at Ser8 or Ser283. Although we were unable to generate a functional phosho-Ser283 antibody, the Ser8 phospho-specific antibody worked well and was fully phospho-specific, provided that the dephosphopeptide was included in the incubations with the primary antibody (i.e. it did not cross-react with the non-phosphorylated peptide; Fig. 6A). It was therefore suitable for use to study the phosphorylation of Ser8 in PSF in HEK293 cells. The antibody detected a band in the position of PSF (as confirmed using anti-PSF antiserum, Fig. 6B). The signal detected in serum-starved cells was not increased by treatment with the phorbol ester TPA (which activates ERK signaling (36)). This is consistent with this site being phosphorylated by Mnk2, which has high basal activity that is not increased much by TPA treatment (16). Also consistent with this, the signal was essentially eliminated by treating the

![Image](https://example.com/figure6.png)
cells with the Mnk1/2-specific inhibitor CGP57380 (37) (Fig. 6B). As a further test of the specificity of the anti-Ser(P)8 antibody, we performed immunoprecipitations either with this antibody or with anti-PSF, and analyzed the immunoprecipitates by Western blot using the other antibody. As shown in Fig. 6C, in each case a single band was observed that migrated at the expected position for PSF, and which was not observed in control immunoprecipitations (using an isotype-matched negative control antibody). This confirms that the phosphospecific antibody is indeed specific for PSF.

We used the Mnk inhibitor CGP57380 to study whether phosphorylation of PSF affected its interactions. Treatment of Jurkat cells with αCD3/αCD28 and/or CGP57380 did not alter the binding of PSF to p54

PSF may be important in regulating such mRNAs. Importantly, we show that PSF associates specifically with the TNFα mRNA, but not to a "negative control," the GAPDH mRNA, in living cells. It is likely that this specificity is conferred by the ARE in the 3′-untranslated region of the TNFα mRNA. Our initial identification of PSF as a novel substrate for the Mnks and as binding to mRNAs, such as that for TNFα, that possess AREs. We show that PSF binds to mRNAs that contain AREs, demonstrating a specific function for PSF-p54

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Here, we identify two sites in PSF, Ser

and Ser

PSF and p54nrb may be important in regulating such mRNAs. Importantly, we show that PSF associates specifically with the TNFα mRNA, but not to a "negative control," the GAPDH mRNA, in living cells. It is likely that this specificity is conferred by the ARE in the 3′-untranslated region of the TNFα mRNA. Our initial identification of PSF as a novel substrate for the Mnks and as binding to mRNAs, such as that for TNFα, that possess AREs. We show that PSF binds to mRNAs that contain AREs, demonstrating a specific function for PSF-p54nrb complexes. Indeed, PSF and p54nrb bind to several oligoribonucleotides that are based on ARE-containing mRNAs including ones encoding cytokines, immediate early genes, or other proteins involved in inflammation.

FIGURE 7. Mnk inhibition does not affect the interaction between PSF and p54

We used the Mnk inhibitor CGP57380 to study whether phosphorylation of PSF affected its interactions. Treatment of Jurkat cells with αCD3/αCD28 and/or CGP57380 did not alter the binding of PSF to p54

as assessed by standard (Fig. 7C) or Q-PCR (Fig. 7D, left-hand panel). As reported earlier, CGP57380 did not affect total TNFα mRNA levels at 45 min before stimulation. After 1 h of stimulation, cells were harvested and anti-PSF-coated protein G beads were used to immunoprecipitate PSF from the cell extracts. The presence of PSF and p54nrb in the pull-downs was analyzed by Western blot using the other antibody. As shown in Fig. 7E, lysates were prepared and fractionated. Total RNA was extracted from 100 μg of nuclear or cytoplasmic fractions using the TRIzol method. RNA levels were analyzed by RT Q-PCR. E, Jurkat cells were treated as indicated. Lysates were then prepared and fractionated. Total RNA was extracted from 100 μg of nuclear or cytoplasmic (C) fractions using the TRIzol method. RNA levels were analyzed by RT Q-PCR.

PSF and p54nrb may be important in regulating such mRNAs. Importantly, we show that PSF associates specifically with the TNFα mRNA, but not to a "negative control," the GAPDH mRNA, in living cells. It is likely that this specificity is conferred by the ARE in the 3′-untranslated region of the TNFα mRNA. Our initial identification of PSF as a novel substrate for the Mnks and as binding to mRNAs, such as that for TNFα, that possess AREs. We show that PSF binds to mRNAs that contain AREs, demonstrating a specific function for PSF-p54nrb complexes. Indeed, PSF and p54nrb bind to several oligoribonucleotides that are based on ARE-containing mRNAs including ones encoding cytokines, immediate early genes, or other proteins involved in inflammation.
cells, and that its phosphorylation is blocked by the Mnk inhibitor CGP57380, thus identifying PSF as a new intracellular Mnk substrate. Interestingly, Ser8 was preferentially phosphorylated by Mnk2b, providing the first evidence for differences between the specificities of Mnk1 and Mnk2. Few Mnk substrates are so far known, and most are involved in mRNA translation or the nuclear processing of such mRNAs or their transport into the cytoplasm.

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