Multiple interactions between RNA polymerase I, TIF-IA and TAF_I subunits regulate preinitiation complex assembly at the ribosomal gene promoter

Xuejun Yuan, Jian Zhao, Hanswalter Zentgraf¹, Urs Hoffmann-Rohrer & Ingrid Grummt⁺

Division of Molecular Biology of the Cell II and ¹Applied Tumor Virology, German Cancer Research Center, D-69120 Heidelberg, Germany

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In mammals, growth-dependent regulation of rRNA synthesis is brought about by the transcription initiation factor TIF-IA. TIF-IA is associated with a fraction of the TBP-containing factor TIF-IB/SL1 and the initiation-competent form of RNA polymerase I (Pol I). We investigated the mechanisms that down-regulate cellular pre-rRNA synthesis and demonstrate that nutrient starvation, density arrest and protein synthesis inhibitors inactivate TIF-IA and impair the association of TIF-IA with Pol I. Moreover, we used a panel of TIF-IA deletion mutants to map the domains that mediate the interaction of TIF-IA with Pol I and TIF-IB/SL1. We found that amino acids 512–609 interact with two subunits of Pol I, RPA43 and PAF67, whereas a short, conserved motif (LARAK, amino acids 411–415) is required for the association of TIF-IA with TAF₉₅ and TAF₆₈. The results uncover an interphase for essential protein–protein interactions that facilitate Pol I preinitiation complex formation.

INTRODUCTION

Preinitiation complex formation at the mammalian ribosomal RNA gene promoter is nucleated by the synergistic action of two DNA binding proteins, the HMG-box-containing upstream binding factor UBF (Jantzen *et al.*, 1992) and the RNA polymerase I (Pol I)-specific TBP–TAF_I complex TIF-IB/SL1 (Comai *et al.*, 1992; Eberhard *et al.*, 1993). Pol I, together with two associated initiation factors, TIF-IA and TIF-IC, is recruited to the transcription start site by specific interaction with UBF and TIF-IB/SL1 bound to the core element of the rDNA promoter (for a review, see Grummt, 1999). TIF-IA was initially characterized as an activity that complements inactive extracts from quiescent mouse cells (Buttgereit *et al.*, 1985). Extracts prepared from

serum-starved or cycloheximide-treated cells lack this activity and are therefore transcriptionally inactive. TIF-IA corresponds to factor C* (Brun *et al.*, 1994) and yeast Rn3p (Yamamoto *et al.*, 1996). Both in yeast and mammals, Rn3p/TIF-IA associates with a subpopulation of Pol I to form the transcriptionally active enzyme, defined as the Pol I entity that is capable of initiating transcription from the rDNA promoter.

Besides being associated with Pol I, TIF-IA has been shown to interact with TIF-IB/SL1 (Miller *et al.*, 2001). This suggests that, by interacting with both Pol I and TIF-IB/SL1, TIF-IA targets transcriptionally active Pol I molecules to the rDNA promoter. Given the essential role of TIF-IA in adapting rDNA transcription to cell growth, we undertook a detailed analysis of the protein domains that mediate the interaction of TIF-IA with Pol I and TIF-IB/SL1. We demonstrate that the C-terminal part of TIF-IA interacts with two subunits of Pol I, RPA43 and PAF67, whereas an internal region of TIF-IA (LARAK, amino acids 411–415) mediates the interaction with TAF₆₈ and TAF₉₅. Importantly, the interaction between TIF-IA and Pol I is impaired in stationary, starved and cycloheximide-treated cells, underscoring the biological significance of these protein–protein interactions in initiation complex formation.

RESULTS AND DISCUSSION

TIF-IA activity is down-regulated in growth-arrested cells

Fluctuations in cellular rRNA synthesis have been observed under a variety of conditions that affect cell growth and metabolism. Previous studies revealed that the shutdown of rRNA synthesis

⁺Corresponding author. Molecular Biology of the Cell II, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel: +49 6221 423423; Fax: +49 6221 423404; E-mail: i.grummt@dkfz.de

Fig. 1. TIF-IA is inactivated in growth-arrested cells. (A) Northern blot. 45S pre-rRNA levels were monitored in 15 μ g of RNA from exponentially growing, serum-starved, cycloheximide-treated and density-arrested cells. To normalize for variations of RNA loading, the blot was also hybridized with a probe complementary to cytochrome C oxidase (cox) mRNA. (B) Western blot. TIF-IA and Pol I from exponentially growing (lane 1), density-arrested (lane 2), amino-acid-starved (lane 3) or cycloheximide-treated (lane 4) FM3A cells were visualized on immunoblots with α -TIF-IA or α -RPA116 antibodies. (C) *In vitro* transcription. Nuclear extract proteins (50 μ g) from growing (lane 1), density-arrested (lanes 2 and 5), cycloheximide-treated (lanes 3 and 6) and amino-acid-starved (lanes 4 and 7) FM3A cells were assayed for transcriptional activity in the absence (lanes 1–4) or presence (lanes 5–7) of 30 ng of recombinant TIF-IA. (D) TIF-IA is targeted by extracellular signals that inhibit growth. TIF-IA (30 and 60 ng) purified from exponentially growing (lanes 2 and 3), density-arrested (lanes 4 and 5), amino-acid-starved (lanes 6 and 7) and cycloheximide-treated (lanes 8 and 9) NIH 3T3 cells were assayed for their capability to restore the transcriptional activity of a nuclear extract from density-arrested FM3A cells (lane 1). (E) The interaction between TIF-IA and Pol I is impaired in growth-arrested cells. NIH 3T3 cells (1×10^6) were transfected with 2 μ g of pcDNA3.1-Flag-hTIF-IA. To achieve confluency, 8×10^6 cells were seeded onto a 100 mm plate 12 h after transfection. Alternatively, cells were treated with cycloheximide or starved of cystine and methionine for 2 h before harvesting. Pol I was immunoprecipitated from cell lysates with α -RPA53 antibodies, and TIF-IA and Pol I were detected on western blots.

during mitosis and early G₁ phase is due to inactivation of TIF-IB/SL1 and UBF (Heix *et al.*, 1998; Klein and Grummt, 1999). Growth-dependent regulation of Pol I, on the other hand, has been attributed to alterations in the amount or activity of TIF-IA (Buttgereit *et al.*, 1985; Schnapp *et al.*, 1993). To study the molecular mechanisms mediating growth-dependent regulation of Pol I transcription, pre-rRNA levels of exponentially growing NIH 3T3 cells were compared with those of serum-starved, cycloheximide-treated and density-arrested cells. Pre-rRNA synthesis was monitored on northern blots using a labeled probe that hybridizes to the 5' end of 45S pre-rRNA and specifically detects unprocessed pre-rRNA molecules. Figure 1A shows that pre-rRNA synthesis was markedly decreased in serum-starved, cycloheximide-treated and density-arrested cells. This reduction of rRNA synthetic activity supports early studies demonstrating that conditions that harm cellular metabolism impair Pol I transcription (Yu and Feigelson, 1972).

To investigate whether the amount or activity of TIF-IA was altered in response to changes in cell growth, TIF-IA levels and transcriptional activity were compared in nuclear extracts from density-arrested, amino-acid-starved or cycloheximide-treated cells. All four extracts contained similar levels of TIF-IA and Pol I (Figure 1B) but exhibited marked differences in transcriptional activity. Extracts from growing cells efficiently transcribed rDNA (Figure 1C, lane 1), whereas extracts from dense, cycloheximide-

treated or starved cells were virtually inactive (lanes 2–4). Significantly, the addition of recombinant TIF-IA restored transcriptional activity, reaching levels that are comparable to the control extract (lanes 5–7). This result demonstrates that TIF-IA, but none of the other components of the Pol I transcription machinery, is targeted by diverse signaling pathways that ultimately down-regulate the cell's biosynthetic activity.

If this conclusion is correct, then TIF-IA from growth-arrested cells should be transcriptionally inactive. To test this, TIF-IA was immunopurified from growing, confluent, starved and cycloheximide-treated NIH 3T3 cells, and equal amounts of TIF-IA were assayed for their capability to restore Pol I transcription in extracts from stationary cells. TIF-IA from growing cells activated Pol I transcription (Figure 1D, lanes 2 and 3). In contrast, TIF-IA from density-arrested, starved and cycloheximide-treated cells was transcriptionally inactive (lanes 4–9). Thus, the activity, but not the amount, of TIF-IA is regulated by signals that impair cell metabolism and growth.

TIF-IA has been shown to be part of the Pol I 'holoenzyme', the enzyme moiety that is associated with most, if not all, factors required for transcription initiation (Saez-Vasquez and Pikaard, 1997; Seither *et al.*, 1998; Albert *et al.*, 1999). Moreover, TIF-IA has been shown to interact with TIF-IB/SL1 (Miller *et al.*, 2001), which suggests that TIF-IA bridges both protein complexes. To assess the function of TIF-IA in initiation complex formation, Pol I

Fig. 2. The C-terminus of TIF-IA interacts with RPA43 and PAF67. (A) Interaction of TIF-IA with Pol I and TIF-IB/SL1. Recombinant TIF-IA was incubated with 50 μ l of partially purified cellular Pol I or TIF-IB/SL1 and incubated with control beads (lane 2) or bead-bound α -TIF-IA antibodies (lane 3). Associated Pol I and TIF-IB were visualized on western blots using α -RPA116 or α -TAF95 antibodies. (B) GST pull-down assays. The indicated GST fusion proteins were immobilized on glutathione-Sepharose and incubated with ³⁵S-labeled TIF-IA. TIF-IA bound to GST (lane 2), GST-RPA43 (lane 3), GST-RPA53 (lane 4) and GST-PAF67 (lane 5) was visualized by autoradiography. (C) RPA43 and PAF67 form a stable complex with TIF-IA. Extracts from *E. coli* expressing TIF-IA and GST (lane 1), TIF-IA and GST-RPA43 (lane 2), TIF-IA and GST-RPA53 (lane 3) or TIF-IA and GST-PAF67 (lane 4) were assayed on western blots for TIF-IA levels (lower panel). After incubation with glutathione-Sepharose, associated TIF-IA was monitored on western blots (upper panel). (D) Mutational analysis of TIF-IA/Pol I interactions. ³⁵S-labeled TIF-IA and the respective mutants were incubated with immobilized GST (lanes 2 and 5), GST-RPA43 (lane 3) and GST-PAF67 (lane 6). TIF-IA was analyzed by SDS-PAGE and autoradiography. Twenty percent of the input proteins are shown in lanes 1 and 4.

was precipitated from exponentially growing, confluent, starved or cycloheximide-treated cells, and coprecipitated TIF-IA was detected on western blots. Consistent with TIF-IA being associated with Pol I, significant amounts of TIF-IA were present in the immunoprecipitates from growing cells (Figure 1E, lane 5). Importantly, the amount of TIF-IA that was associated with Pol I markedly decreased in growth-arrested cells (lanes 6–8). This result is consistent with recent data in yeast and mammals demonstrating dissociation of TIF-IA from Pol I in stationary or cycloheximide-treated cells (Milkereit and Tschochner, 1998; Cavanaugh *et al.*, 2002). The current view is that inhibition of cell growth leads to hypophosphorylation and inactivation of TIF-IA and impairs the association of TIF-IA with Pol I.

TIF-IA interacts with two subunits of Pol I

To study the interaction of TIF-IA with Pol I and TIF-IB/SL1, Flag-tagged TIF-IA was incubated with cellular fractions containing partially purified Pol I or TIF-IB/SL1, precipitated with anti-FLAG antibodies, and coprecipitated Pol I and TIF-IB/SL1 were analyzed on western blots. Consistent with TIF-IA mediating the interaction between Pol I and TIF-IB/SL1, both Pol I and TIF-IB/SL1 coprecipitated with TIF-IA (Figure 2A).

In *Saccharomyces cerevisiae*, Rn3p has been found to interact with RPA43, a unique subunit of Pol I (Peyroche *et al.*,

2000; Fath *et al.*, 2001). Given that functionally important protein–protein interactions are evolutionarily conserved, we examined whether the homologous mammalian subunit mediates the interaction with TIF-IA. To monitor interactions between TIF-IA and subunits of Pol I, pull-down experiments were performed (Figure 2B). ³⁵S-labeled TIF-IA interacted with GST–mRPA43 but not with GST (lanes 2 and 3). No interaction was observed with RPA53, the third largest subunit of Pol I (lane 4). Surprisingly, the strongest binding was observed to PAF67 (lane 5), a 67 kDa protein that is tightly associated with the initiation-competent form of Pol I and was suggested to play an essential role in recruiting Pol I to the rDNA promoter (Seither *et al.*, 2001).

The interaction of TIF-IA with both PAF67 and RPA43 was substantiated by co-expressing TIF-IA in *Escherichia coli* together with GST fusion proteins encoding PAF67, RPA53 or mRPA43 and capturing bound TIF-IA on glutathione-Sepharose. Consistent with the results above, TIF-IA was bound to GST–PAF67 and GST–RPA43 but not to control beads or GST–RPA53 (Figure 2C). Again, TIF-IA bound more efficiently to GST–PAF67 than to GST–RPA43. Thus, TIF-IA interacts with two polypeptides of Pol I, e.g. RPA43, a genuine subunit and PAF67, a Pol I-associated factor that marks the initiation-competent enzyme moiety. This suggests that, by interacting with PAF67, TIF-IA may target a functional subset of Pol I molecules into the initiation complex.

Fig. 3. A conserved motif (LARAK) mediates the interaction of TIF-IA with TAF₉₅ and TAF₆₈. (A) Pull-down assay. Bead-bound Flag-tagged TIF-IA was incubated with extracts from Sf9 cells overexpressing HA-tagged TAF₄₈, TAF₆₈, TAF₉₅ or TBP, respectively. Proteins were separated by SDS-PAGE, and TAFs were visualized on western blots using α -HA antibodies (lane 3). M2 beads saturated with the Flag epitope peptide were used as a control (lane 2). (B) The LARAK motif mediates the interaction of TIF-IA with TAF₆₈ and TAF₉₅. Flag-tagged TIF-IA (lane 3), TIF-IA Δ 377–512 (lane 4), TIF-IA Δ 95–163 (lane 5) and TIF-IA_{LARAK} (lane 6) were incubated with HA-tagged TAF₆₈ or TAF₉₅. TIF-IA was immunoprecipitated, and associated TAFs were visualized on western blots. A scheme of the mutants and a sequence alignment of the LARAK domain of TIF-IA homologues are shown above. H.s., *Homo sapiens*; M.m., *Mus musculus*; S.p., *Schizosaccharomyces pombe*; S.c., *Saccharomyces cerevisiae*; C.e., *Caenorhabditis elegans*. (C) A synthetic peptide (LARAK) blocks the interaction between TIF-IA and TIF-IB/SL1. Immobilized TIF-IA was incubated at 4°C for 4 h with partially purified TIF-IB/SL1 in the absence (lane 3) or presence of 200 ng control peptide (EHLWKKLQDPSNPAI, lane 4) or LARAK peptide (YIGSFLARAKFITVKSC, lane 5) in 250 μ l of buffer AM-100/0.5% NP-40. After stringent washing, bound TIF-IB/SL1 was analyzed on immunoblots with α -TAF₉₅ or α -TAF₆₈ antibodies. (D) The LARAK motif is essential for TIF-IA activity. *In vitro* transcription assays contained nuclear extracts from density-arrested FM3A cells and no exogenous TIF-IA (lane 1) or 15, 30 and 50 ng of wild-type TIF-IA (lanes 2–4) or TIF-IA_{LARAK} (lanes 5–7). The amounts of recombinant TIF-IA added to the reactions was monitored on western blots with α -Flag antibodies (lower panel). (E) Deletion or permutation of the LARAK motif abrogates TIF-IA activity *in vivo*. NIH 3T3 cells were cotransfected with 2.5 μ g of the rDNA reporter plasmid and 1 or 2 μ g of pBK-CMV-Flag-TIF-IA (lanes 2 and 3), pBK-CMV-Flag-TIF-IA_{LARAK} (lanes 4 and 5) or pBK-CMV-Flag-TIF-IA_{LARAK} (lanes 6 and 7). Transcripts from the rDNA reporter were monitored on northern blots. TIF-IA expression levels were monitored on western blots with α -Flag antibodies (lower panel).

To map the respective domains of TIF-IA that interact with RPA43 and PAF67, GST pull-down experiments were performed with a set of TIF-IA deletion mutants. As shown in Figure 2D, mutants lacking the N-terminus (TIF-IA/ Δ N94), a large internal region (TIF-IA/ Δ 377–512) or the C-terminal region (TIF-IA/ Δ C609) efficiently associate with both RPA43 and PAF67. Deletion of 95 amino acids from the C-terminus (TIF-IA/ Δ C556) reduces binding to PAF67 without affecting the interaction with RPA43. Further truncation of the C-terminus (TIF-IA/ Δ C499) abolishes binding of TIF-IA to both RPA43 and PAF67. This suggests that two domains located between residues 512 and 609 mediate the interaction between TIF-IA and Pol I.

A conserved sequence element (LARAK) mediates the interaction between TIF-IA and TIF-IB

To examine the interaction of TIF-IA with subunits of TIF-IB, immobilized TIF-IA was incubated with extracts from Sf9 cells that overexpress either of the four subunits of TIF-IB. Bound proteins were analyzed on western blots using antibodies against TAF₉₅, TAF₆₈, TAF₄₈ and TBP, respectively. As demonstrated in Figure 3A, TIF-IA interacted with TAF₉₅ and TAF₆₈, whereas no interaction of TIF-IA with TAF₄₈ or TBP was observed.

To define the domains of TIF-IA that mediate the interaction with TAFs, wild-type and mutant TIF-IA were expressed in Sf9 cells and incubated with cell lysates containing HA-tagged TAF₉₅ or TAF₆₈, and bound TAFs were monitored on immunoblots. Truncation of the N- or C-terminal part of TIF-IA did not affect the interaction with either subunit (data not shown). Similarly, a mutant lacking amino acids 95–163 bound to both subunits with an efficiency comparable with wild-type TIF-IA (Figure 3B, lane 5). In contrast, deletion of residues 377–512 abolished binding (lane 4). A search for conserved sequence elements within amino acids 377–512 revealed a motif (F/Y(L)ARAK) that is highly conserved among different species (Figure 3B). Deletion of the LARAK sequence (amino acids 411–415) abrogated TIF-IA binding to both TAFs (Figure 3B, lane 6), indicating that this motif serves an essential role in preinitiation complex assembly.

Given that the LARAK motif mediates the interaction between TIF-IA and TIF-IB/SL1, a synthetic peptide harboring the LARAK sequence should prevent the interaction of both factors. Indeed, the LARAK peptide blocked the association of TIF-IA with TIF-IB/SL1 (Figure 3C, lane 5), whereas a control peptide had no effect (lane 4). Thus, amino acids 411–415 are required for the association of TIF-IA with TIF-IB.

Consistent with the functional significance of the LARAK motif, TIF-IA_{ΔLARAK} was transcriptionally inactive. TIF-IA_{ΔLARAK} was incapable of complementing inactive extracts from density-arrested cells *in vitro* (Figure 3D). Moreover, when assayed for activation of a Pol I reporter gene *in vivo*, wild-type but not TIF-IA_{ΔLARAK} stimulated Pol I transcription (Figure 3E, lanes 1–5). Converting the LARAK sequence to LRAKA, a permutation that does not alter the charge of TIF-IA, also abolished TIF-IA activity (lanes 6 and 7). These results demonstrate that the interaction between TIF-IA and TIF-IB is mediated by a discrete number of amino acids that have been evolutionary conserved.

The finding that both TIF-IA and the yeast homologue Rrn3 is a key player in recruiting Pol I to the rDNA promoter underscores the essential role of this transcription factor in rRNA synthesis. TIF-IA activity is regulated by diverse extracellular signals, indicating that this factor adapts Pol I transcription to cell growth. TIF-IA is phosphorylated at multiple sites (unpublished data), and data in yeast have revealed that reversible phosphorylation of both Rrn3 and Pol I mediate formation and dissociation of the initiation complex (Fath *et al.*, 2001). Therefore, elucidating the chains of events by which extracellular signals are transferred into the nucleolus and modify the activity of Rrn3/TIF-IA will unravel the mechanism of Pol I transcription initiation and the pathways that control this essential process.

METHODS

Plasmids. The cDNA encoding TIF-IA (DDB/EMBL/GenBank database accession No. AJ272050) was tagged with the Flag-epitope and subcloned into various expression vectors. Mutant forms of TIF-IA were generated by *in vitro* mutagenesis using standard techniques. The rDNA reporter pMr1930-BH has been described previously (Budde and Grummt, 1999). The cDNA encoding amino acids 1–248 of mouse RPA43 was amplified from plasmid IMAGp998M106538 (RZPD).

Cell culture and transfection experiments. HEK 293T or NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. Cells were harvested either in the log-phase or after reaching confluence. Alternatively, cells were starved of cystine and methionine or treated with 0.1 mg/ml cycloheximide for 2 h. For *in vivo* analysis of wild-type and mutant TIF-IA, 5×10^5 cells were cotransfected with 2.5 µg of reporter plasmid (pMr1930-BH) and different amounts of a CMV-based expression vector encoding tagged wild-type or mutant forms of TIF-IA. After 48 h, cellular RNA was isolated and hybridized to ³²P-labeled probes that are complementary to pUC sequences inserted between the rDNA promoter and terminator in pMr1930-BH.

Expression and purification of recombinant TIF-IA. The expression and purification of Flag-tagged TIF-IA from insect cells has been described previously (Bodem *et al.*, 2000). To immunopurify cellular TIF-IA, 5×10^6 NIH 3T3 cells were lysed for 45 min at 4°C in 0.8 ml of 20 mM Tris–HCl pH 7.4, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100 in the presence of protease inhibitors. After centrifugation for 30 min at 10 000 g, the supernatants were incubated for 4 h with α-TIF-IA antibodies (10 µg) bound to 20 µl of protein G-agarose. After washing, the beads were resuspended in 200 µl of buffer AM-100 and assayed for transcriptional activity.

***In vitro* transcription assays.** Standard reactions (25 µl) contained 30 ng of template pMr600/EcoRI, 12 mM Tris–HCl pH 7.9, 0.1 mM EDTA, 0.5 mM dithioerythritol, 5 mM MgCl₂, 80 mM KCl, 12% glycerol, 0.66 mM each of ATP, CTP and GTP, 0.01 mM UTP, 1 µCi [α-³²P]UTP and 50 µg of nuclear extract proteins from FM3A cells.

Immunoprecipitation and immunoblotting. Immunoprecipitations were carried out using nuclear extracts from FM3A cells, lysates from NIH 3T3 cells, or HEK 293T cells overexpressing Flag-tagged TIF-IA. Cells were lysed in 1 ml of immunoprecipitation buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT) and incubated for 4 h at 4°C with the respective antibodies. After extensive washing, precipitated proteins were analyzed on immunoblots.

Protein–protein interaction experiments. Immobilized Flag-tagged TIF-IA and control beads saturated with the Flag-epitope peptide were incubated with lysates from Sf9 cells overexpressing recombinant subunits of TIF-IB. After incubation for 2 h at 4°C in buffer AM-100 plus 0.5% NP40 and washes, associated proteins were identified on western blots. To examine the association of TIF-IA with Pol I and TIF-IB, 500 ng of TIF-IA expressed in Sf9 cells were incubated for 2 h at 4°C with 50 µl of partially purified Pol I or TIF-IB (H-400 or CM-400 fraction; Schnapp and Grummt, 1996). After precipitation with anti-Flag antibodies, associated proteins were identified on western blots. To monitor the interaction between TIF-IA and subunits of Pol I *in vitro*, GST, GST–RPA43, GST–RPA53 and GST–PAF67 were attached to glutathione–Sepharose, and 10 µl of packed beads containing 5 µg of GST or GST fusion proteins were incubated with 7 µl of ³⁵S-labeled TIF-IA in 100 µl of buffer (50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors). After incubation for 1 h at room temperature, beads were washed, and bound proteins were analyzed by SDS–PAGE and autoradiography. Alternatively, TIF-IA and GST fusion proteins were co-expressed in *E. coli* and bound to glutathione–Sepharose as described previously (Peyroche *et al.*, 2000).

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