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Direct interaction of nucleoporin p62 with mRNA during its export from the nucleus

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SUMMARY

Primary transcripts in eukaryotic cells undergo several processing steps within the nucleus, and resulting mature RNA molecules are selectively exported to the cytoplasm. Nucleo-cytoplasmic mRNA transport is an active process that likely involves RNA-protein interactions. To identify specific RNA-binding proteins, we designed a novel approach, which allows the analysis of interactions between mRNAs and proteins along the transport pathway. The method consists of inducing in vivo a covalent binding between nuclear proteins and microinjected mRNAs. Using such a procedure, we were able to detect a direct interaction between nucleoporin p62 with mRNA during export. The formation of the mRNA-p62 complex was inhibited by wheat-germ agglutinin, an inhibitor of mRNA export. Antibodies directed against p62 caused a substantial reduction in the rate of mRNA export from the nucleus.

Key words: mRNA export, nucleoporin, crosslinking

INTRODUCTION

In eukaryotic cells, primary RNA transcripts undergo several processing steps within the nucleus that generate mature RNA molecules. These mRNAs are then selectively transported to the cytoplasm where they fulfill their function. Compared to nuclear import processes, the export of RNAs from the nucleus is still poorly understood.

Using microinjection into *Xenopus laevis* oocytes, the export of mRNA from the nucleus to the cytoplasm has been shown to occur through the nuclear pore complex and to display properties of a saturable and ATP-dependent process facilitated by the presence of the cap structure m^7GpppN at the 5′ end of the RNA (Dargemont and Kühn, 1992). Transport of one mRNA can be inhibited by competition with a second mRNA but not by the same amount of U snRNA, tRNA or 5 S rRNA, indicating that an essential export factor(s) is specifically required for mRNA export (Dargemont and Kühn, 1992; Jarmolowski et al., 1994). These data lead to the conclusion that transport of mRNAs involves their interaction with proteins. Such proteins may be involved at different levels of the export process including recognition of the RNA, its delivery to or acceptance by the transport machinery, and possibly also modification of its folded state prior to the translocation. Components of the transport machinery such as nuclear pore proteins are also candidates for interaction with mRNA.

In the nucleus, precursor and mature forms of mRNAs are associated with highly abundant nuclear proteins as heterogeneous nuclear ribonucleoproteins (Dreyfuss et al., 1993). Some of these proteins have been shown to shuttle between the nucleus and the cytoplasm and consequently it has been suggested that they are exported together with mRNA and perhaps also to be actively involved in the transport process (Pinol-Roma and Dreyfuss, 1992). A nuclear cap-binding protein has been recently purified from HeLa cells and exhibits a specificity for different cap structure analogs that is consistent with the capacity of these analogs to inhibit U1 snRNA export (Izaurralde et al., 1992). Although these RNA-binding proteins represent good candidates for playing an important role in mRNA export from the nucleus, their direct involvement in this process has not yet been proven. Finally, the identification of temperature-sensitive yeast mutants that accumulate poly(A)$^+$ RNA in their nuclei at the restrictive temperature has lead to the identification of genes whose products influence mRNA transport by mechanisms that are currently unclear (Amberg et al., 1992, 1993; Forrester et al., 1992; Kadowaki et al., 1992, 1993).

To study proteins involved in mRNA export, it appeared necessary to set up experimental approaches that allow the detection of RNA-protein interactions occuring along the export pathway in vivo. Using gel retardation assays, we previously reported that mRNA injected into *Xenopus* oocytes nuclei associates with endogenous nuclear proteins but the identity of these proteins, as well as their role in mRNA export, remains unknown (Dargemont and Kühn, 1992). In order to further characterize the RNA-protein interactions involved in the transport process, we designed a method of in vivo crosslinking that allows the detection of low affinity and/or transient complexes between mRNA that have been injected into *Xenopus* oocyte nuclei and endogenous proteins. This

approach allowed us to show that the nucleoporin p62 is involved in mRNA export from the nucleus.

MATERIALS AND METHODS

Animals and reagents

Female *Xenopus laevis* frogs were purchased from the African *Xenopus* Facility (Noodhoek, South Africa). WGA was from Sigma (München, FRG).

Preparation of radiolabeled mRNA

The full-length human transferrin receptor (TfR) cDNA insert of pcD-TR1 (Kühn et al., 1984) was subcloned into pGEM-3Zf(–) (Promega Biotec, Madison, WI) in positive orientation relative to the promoter for T7 RNA polymerase. The plasmid was linearized with *Bam*HI. In vitro transcripts from this vector yield a 5.0 kb TfR mRNA that contains at its 3′ end an 84-nucleotide long poly(A) tail followed by 32 nucleotides of pcD vector sequence (Neupert et al., 1990). The full-length human 4F2 antigen heavy chain cDNA insert of pCD-4F2.A (Teixeira et al., 1987) was subcloned as a *Bam*HI-*Bam*HI fragment into pSP65 (Boehringer Mannheim Biochemicals, Mannheim, Germany) in positive orientation relative to the promoter for SP6 RNA polymerase. The plasmid was linearized with *Hin*dIII. The in vitro transcript of 2.0 kb contains at its 5′ end 100 bases of pcD vector sequences, and at the 3′ end a poly(A) tail of 120 nucleotides followed by 59 nucleotides of vector sequence.

For in vitro transcription, linearized plasmid DNA (1 μg) was incubated for 90 minutes at 37°C in a 20 μl transcription mixture containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 0.5 mM ATP, 25 μM UTP, 25 μM 5-azido-UTP (Radominska and Drake, 1994), 50 μM CTP, 50 μM GTP, 0.5 mM cap dinucleotide m^7GpppG triphosphate, 75 μg/ml BSA, 40 U RNasin (Promega Biotec), 80 μCi [α-^{32}P]CTP (800 Ci/mmol; Amersham), 40 μCi [α-^{32}P]UTP (400 Ci/mmol; NEN), and 20 units of T7 or SP6 polymerase. DNA was then digested with 2 μg of RNase-free DNase I (Worthington, Freehold, NJ) for 30 minutes at 37°C. Water was added to a final volume of 100 μl, proteins were extracted with phenol-chloroform, unincorporated nucleotides were removed using a Sephadex G-50 column and the RNA was concentrated in 10 μl water (about 200 ng/μl).

Intranuclear microinjection and in vivo crosslinking

Stage VI oocytes were prepared from ovaries of *Xenopus laevis* females defolliculated as described by Gurdon and Wickens (1983) and incubated in modified Barth's solution. A 10 to 20 nl sample of solution containing the mRNA, together with Bromophenol Blue to monitor the actual site of injection, was microinjected per germinal vesicle. Oocytes were maintained at 20°C in Barth's solution and subsequently exposed to 290-310 nm UV light under a bank of 6 Philips TL 40W/01 tubes (courtesy of R. Tyrrell, ISREC, Lausanne) at a minimal distance, for 2 minutes on ice. Oocytes were manually dissected at 4°C in 10 mM HEPES, pH 7.5, 40 mM KCl, 3 mM $MgCl_2$, 10% glycerol and protease inhibitors (aprotinin, 0.5 μg/ml; leupeptin, 1 μg/ml; pepstatin, 0.7 μg/ml; and antipain, 0.7 μg/ml). The nuclear fractions were then digested in dissection buffer for 1 hour at 37°C with RNase T_1 (2 units/nucleus), RNase A (4 μg/nucleus) and DNase I (0.1 units/nucleus) and extracted with 1% MEGA 10 (Boehringer Mannheim Biochemicals) and 100 mM KCl for 30 minutes at room temperature. Samples were analyzed on a 6-15% gradient SDS-PAGE with the equivalent of five oocytes in each lane.

In order to study the effect of anti-p62 antibodies on mRNA export, the immunoglobulin G fraction of the guinea pig serum containing the anti-p62 antibodies (antibody 2; Cordes et al., 1991) was obtained by precipitation in 24.3% ammonium sulfate, pH 7.4. The pellet was resuspended in PBS and dialyzed extensively in PBS. These antibodies (1 mg/ml) were injected 1 hour before mRNA. After mRNA injection, oocytes were fixed in 3% acetic acid, 80% ethanol at 4°C and dissected. The radioactivity present in the nucleus and cytoplasm of each single oocyte was determined, and the percentage of radioactivity present in the nucleus was calculated. Each point represents the average of three experiments and in each experiment six to eight oocytes were injected per condition.

2D Gel analysis

The equivalent of 40 oocyte nuclei were analyzed by isoelectric focusing in the first and SDS-PAGE in the second dimension. For immunoblotting, proteins were transferred to nitrocellulose membranes. Filters were incubated with rabbit antibodies directed against the N terminus of *Xenopus* p62 (antibody 1; Cordes and Krohne, unpublished data) in a dilution of 1:1000, followed by an incubation with anti-rabbit antibodies coupled to horseradish peroxidase and finally developed by using the chemiluminescence (ECL) Western blotting reagents (Amersham) following the manufacturer's instructions.

Immunoprecipitation

Nuclear extracts prepared as described before were diluted in IPP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.25% MEGA 10, 3 mM $MgCl_2$) and precleared by addition of Protein A-Sepharose for 30 minutes at 4°C and centrifuged at 12,000 ***g*** for 2 minutes. The resulting supernatant was incubated overnight at 4°C with Protein A-Sepharose precoated with antibodies 2. Beads were washed twice in IPP buffer, once with the same buffer without MEGA 10 and once with 50 mM Tris-HCl, pH 7.5, boiled in sample buffer and analyzed by SDS-PAGE on 6% to 15% gradient gels.

RESULTS

Detection of complexes between nuclear *Xenopus* oocyte proteins and microinjected mRNA by in vivo UV-crosslinking

In order to generate covalent mRNA-protein interactions, *Xenopus laevis* oocytes were irradiated by UV light after microinjection of radiolabeled mRNAs into their nuclei. The yield of the crosslinked products was increased by using mRNAs containing the photoreactive nucleotide analog, 5-azido-uridine 5′-triphosphate (5-azido-UTP), that can be incorporated during in vitro transcription. In order to be detected, endogenous proteins should therefore bind at least one radioactive nucleotide (UTP or CTP) and one 5-azido-UTP. Oocyte nuclei were subsequently isolated and mRNA complexes analyzed by SDS-PAGE after digestion with RNases A and T_1. The experimental approach is illustrated schematically on Fig. 1.

Using this method, nuclear proteins of between 20 and 200 kDa were cross-linked to transferrin receptor (TfR) mRNA 1 minute after microinjection (Fig. 2A, lane a). The same protein pattern was observed using 4F2 antigen heavy chain mRNA (Fig. 2A, lane b), which shares no sequence homology, and also with TfR mRNA lacking the 3′ UTR and the poly(A) tail (data not shown), suggesting that these proteins are not specific for a given mRNA sequence. In order to follow the dynamics of these interactions during mRNA export, oocytes were irradiated at different time-points after nuclear injection of TfR mRNA. Kinetics of export was measured (Fig. 2C) and RNA-protein complexes from the same number of nuclei were analyzed by SDS-PAGE (Fig. 2B). Since TfR mRNA is being transported to the cytoplasm, nuclei contained a decreasing

amount of labeled mRNA and therefore a decreasing amount of crosslinked product over time. However, while 50% of the microinjected mRNA was exported within about 5 minutes (Fig. 2C; Dargemont and Kühn, 1992), the half-time of disappearence of the complexes varied from 1 to 5 minutes, suggesting a sequential disassembly of these interactions during mRNA transport.

Effect of WGA on the formation of mRNA-protein complexes

Whether any of these RNA-protein interactions are directly involved in mRNA export was not clear. One way to approach this question was to compare the nuclear proteins detected by UV-crosslinking in the normal situation and in conditions known to prevent mRNA transport. We have recently reported that mRNA export is blocked by prior injection of wheat germ agglutinin (WGA) into nuclei (Dargemont and Kühn, 1992) and this inhibition is abolished by co-injection of *N*-acetylglucosamine. We therefore analyzed the effect of nuclear preinjection of WGA on the formation of RNA-protein complexes by the photochemical crosslinking approach (Fig. 3). As compared to a control (lane a), the same proteins were crosslinked in the presence of WGA (lane b) but the intensity of a 62-64 kDa band (indicated by an arrow) was significantly decreased (30% of the control, see figure legend for quantification). It is noteworthy that WGA also seemed to affect the formation of the 85-90 kDa doublet but this effect was weaker and less reproducible. The formation of the 62 kDa complex was not affected when WGA was co-injected with *N*-acetylglucosamine (lane c), indicating that the lectin specifically inhibits the interaction between mRNA and the 62-64 kDa protein.

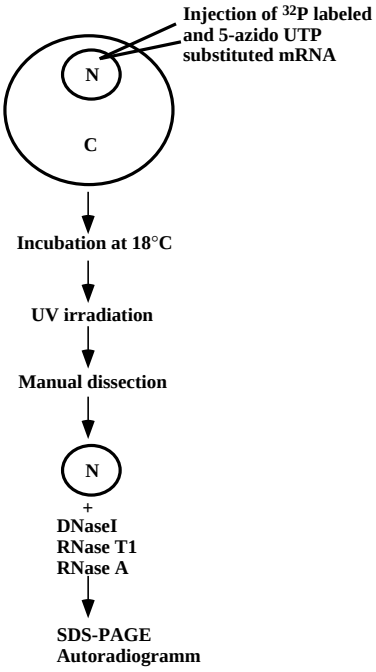

Fig. 1. Scheme of the assay used to induce covalent complexes between mRNAs injected into *Xenopus* oocyte nuclei and endogenous proteins.

Nucleoporin p62 associates with mRNA

WGA is not only able to prevent mRNA export but also to inhibit ribosomal subunit export from the nucleus (Bataille et al., 1990) and protein import into the nucleus (Finlay et al.,

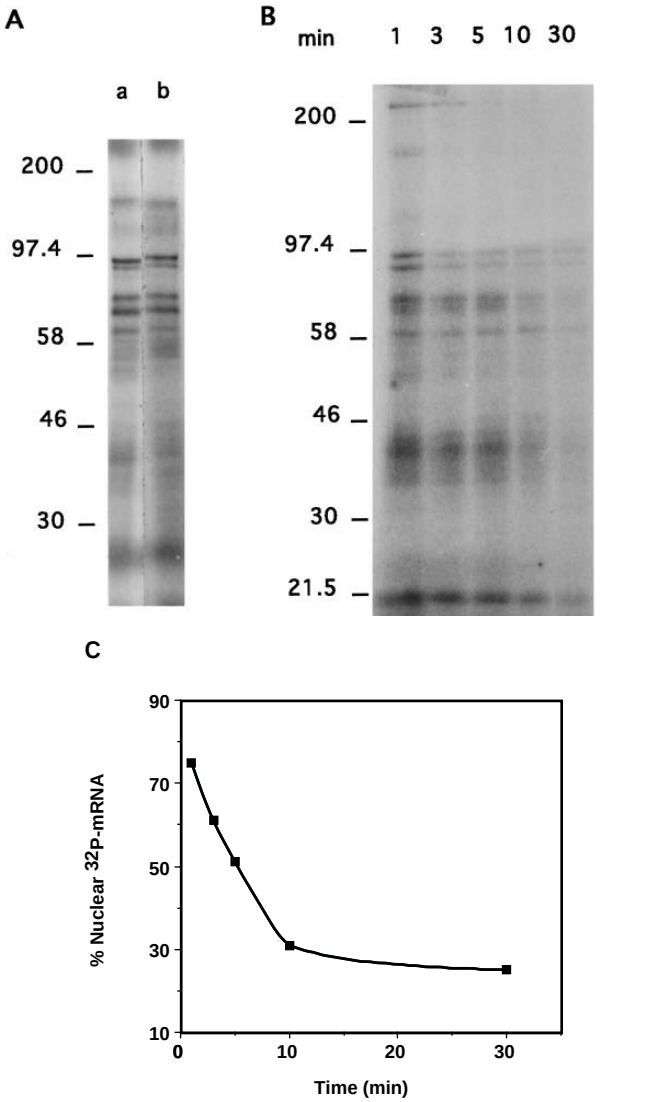

Fig. 2. Detection of complexes between *Xenopus* oocyte nuclear proteins and microinjected mRNA by in vivo UV-crosslinking. (A) A 4 ng or 2 ng sample of ^{32}P-labeled TfR mRNA (a) or 4F2 antigen mRNA (b) respectively, containing 5-azido-UTP was injected into oocyte nuclei. Oocytes were transferred 1 minute after injection to 4°C in order to block mRNA export and then exposed to UV light for 2 minutes. Nuclei were subsequently isolated, homogenized and digested with RNases T_1 and A and DNase I. Nuclear fractions were then extracted in 1% MEGA 10, the RNA-protein complexes resolved in a 10% SDS-PAGE and revealed by autoradiography. (B) A 4 ng sample of ^{32}P-labeled TfR mRNA containing 5-azido-UTP was injected into oocyte nuclei. At different times after injection (1, 3, 5, 10 and 30 minutes), oocytes were transferred to 4°C and irradiated. Nuclear RNA-protein complexes were extracted as described for A and analyzed by 6% to 15% gradient SDS-PAGE. (C) Oocytes treated as described for B were manually dissected, the radioactivity present in the nucleus and cytoplasm of each single oocyte was determined and the percentage of radioactivity present in the nucleus calculated.

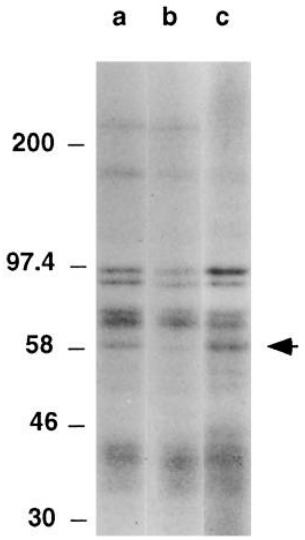

Fig. 3. Effect of WGA on the formation of mRNA-protein complexes. A 4 ng sample of ^{32}P-labeled TfR mRNA was injected into oocyte nuclei that had been pre-injected 1 hour before with water (lane a), with WGA (at a final nuclear concentration of 0.5 mg/ml; lane b) or with WGA plus *N*-acetylglucosamine (50 mM final concentration; lane c). Oocytes were incubated for 2 minute before UV irradiation. Nuclear protein-mRNA complexes were analyzed as described in Fig. 1. The arrow indicates the 62-64 kDa complex, which is significantly diminished in the presence of WGA: after correction for the reduced quantity of the crosslinked complexes in the presence of WGA, the reduction intensity of the 62-64 kDa band was to 30% of the control (lane a). The fact that the intensity of the complexes generally decreased in the presence of WGA and increased in the presence of *N*-acetylglucosamine reflects only the variability of the crosslinking efficiency and not a specific effect of these reagents.

1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Wolff et al., 1988). WGA appears to block protein translocation through the nuclear pore by binding to *N*-acetylglucosaminylated nucleoporins, the most prominent of which is the 62 kDa pore protein, p62 (Finlay et al., 1987; Davis and Blobel, 1986, 1987; Finlay and Forbes, 1990). It was therefore important to determine whether the 62-64 kDa crosslinked product was p62.

For this purpose, radiolabelled crosslinked complexes were submitted to gel electrophoresis and Western blotting with rabbit antibodies directed against the N terminus of *Xenopus* p62 (antibody 1). The radiolabelled 62 kDa crosslinked product was found to co-migrate with p62 (Fig. 4A, see the arrow), suggesting that p62 was the 62 kDa protein crosslinked to mRNA. Another protein or a degradation product of p62 was detected by this antibody (indicated by the star), which migrated clearly not at the correct molecular mass (about 40-45 kDa) and pI of the complete p62 (Fig. 4C; pI about 6.4). In order to show that p62 interacts with mRNA in vivo, nuclear extracts obtained after mRNA injection, UV irradiation and RNA digestion were treated with MEGA 10, a detergent described as able to partially solubilize the nuclear pores (Finlay and Forbes, 1990), and immunoprecipitated with an anti-p62 antibody. Since antibody 1 does not efficiently immunoprecipitates, we used an antibody that has been prepared against recombinant mouse p62 produced in *Escherichia coli*, i.e. a non-glycosylated form of p62 (antibody 2; Cordes et al., 1991). In western blot analysis, this antibody only recognized p62 (data not shown) but was less sensitive in this assay than the antibody 1. Despite a low efficiency of immunoprecipitation due to an incomplete extraction of nuclear pore proteins, an RNA-protein complex of about 62 kDa was specifically precipitated by the anti-p62 antibody (Fig. 4B). The immunoprecipitated complex corresponded in size to the WGA-sensitive interaction shown in Fig. 3. In order to confirm that p62 interacts with mRNA, nuclear extracts obtained after mRNA injection, either with or without subsequent UV irradiation were submitted to RNase and DNase digestion and two-dimensional gel electrophoresis. These gels were subsequently immunoblotted with antibodies directed against p62 (antibody 1). A single isoelectric variant of p62 (pI about 5; Cordes et al., 1991) was revealed in nuclear extracts prepared from non-irradiated oocytes (Fig. 4C, a and b, marked by arrowheads). Two additional variants of p62 appeared when oocytes were exposed to UV light after mRNA injection (Fig. 4C, c and d, see arrowheads). One of these was prominent and the second more acidic spot corresponded to a small fraction of nuclear p62 still detectable by Coomassie Blue staining (Fig. 4C, c). It is noteworthy that the nuclear protein pattern revealed by the Coomassie staining was not affected by the irradiation. Therefore it is unlikely that the two additional variants of p62, which appeared exclusively in irradiated oocytes, resulted from a direct effect of UV light on this protein. Moreover, their more acidic pI values and slightly higher molecular mass than the normal form of p62 (Fig. 4C, a and b) indicated that these spots resulted from cross-linking between p62 and mRNA. In *Xenopus* oocyte nuclei, p62 is exclusively localized to the pores and excluded from the nucleoplasm (Cordes et al., 1991). Thus, the interaction detected by in vivo crosslinking between p62 and mRNA most likely occurs at the nuclear pores.

Inhibition of mRNA export by anti-p62 antibodies

In order to investigate whether p62 not only interacts with mRNA but is also functionally involved in its export from the nucleus, we analyzed the effect of anti-p62 antibodies on mRNA transport. For this purpose, we used the anti-p62, antibody 2, which does not crossreact with any other protein of the nuclear envelope (Cordes et al., 1991). The results, shown in Fig. 5, indicate that nuclear preinjection of antibodies against p62 (1 mg/ml) decreased the rate of appearance of mRNA in the cytoplasm from 1.2×10^8 molecules transported per minute in the control to 0.24×10^8 molecules per minute. This inhibition was, however, abolished when anti-p62 antibodies were coinjected with recombinant p62 protein (100 μg/ml) produced in *E. coli* (Cordes et al., 1991) (Fig. 5). Such an effect was not observed when non-specific immunoglobulins were used (data not shown). These data indicate that inhibition of transport mediated by the anti-p62 antibodies was specific and support the hypothesis that nucleoporin p62 plays an active role in mRNA export.

DISCUSSION

The involvement of *N*-acetylglucosaminylated pore proteins in the export of RNAs has already been suggested by the inhibition of mRNA (Dargemont and Kühn, 1992), the signal recog-

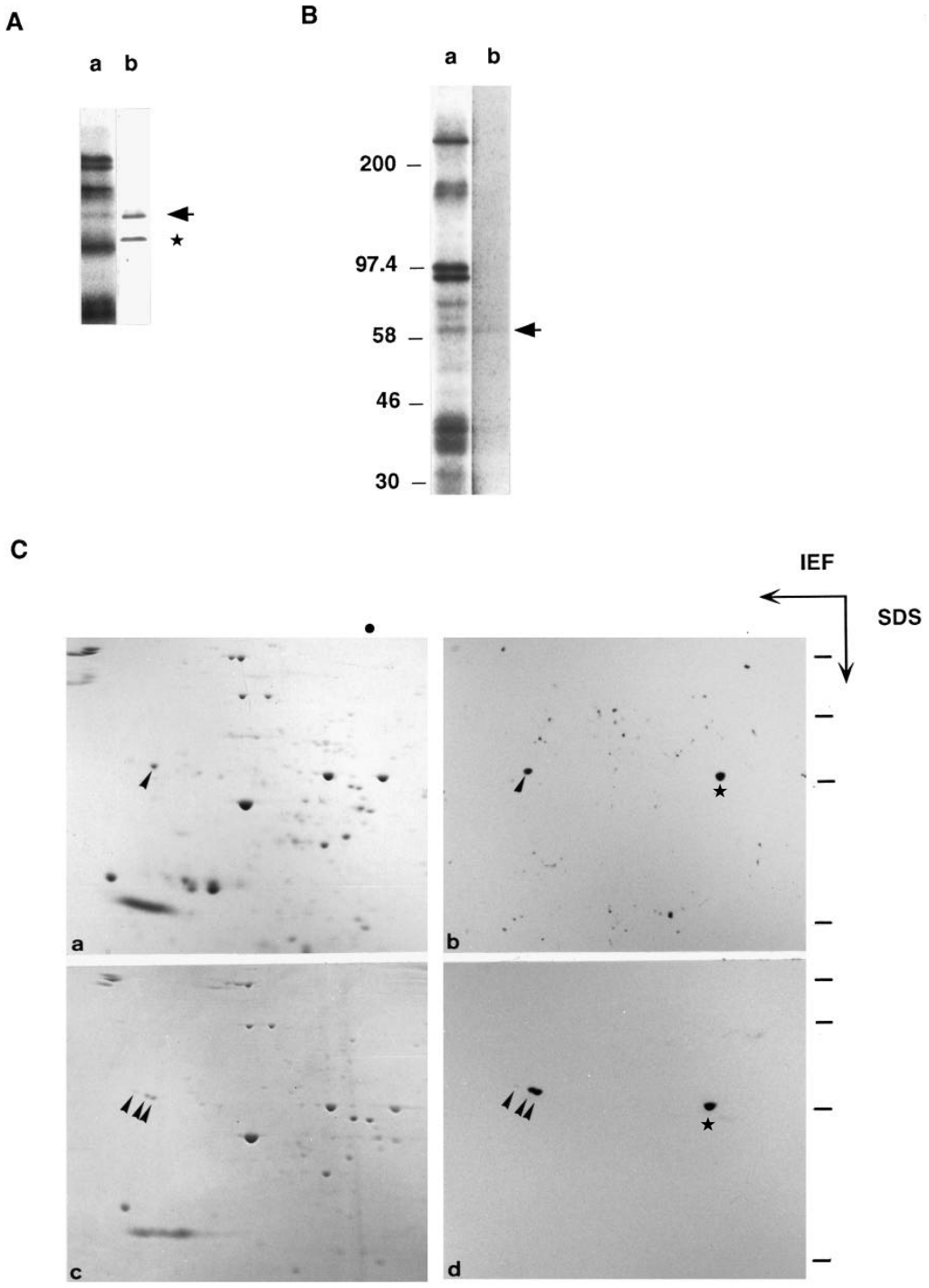

Fig. 4. Nucleoporin p62 interacts with mRNA. (A) Nuclear RNA-protein complexes formed after the nuclear injection of 4 ng of ^{32}P-labeled TfR mRNA and irradiation were extracted as described for Fig. 1, separated on SDS-PAGE (10%) and transferred on nitrocellulose membrane. These complexes were revealed by autoradiography (lane a) and the filter was then blotted with anti-p62 antibodies 1 (lane b). p62 is marked by an arrow whereas the crossreactive band is indicated by a star. (B) Crosslinked RNA-protein complexes from oocyte nuclei injected with 4 ng of ^{32}P-labeled TfR mRNA were extracted as before (lane a) and immunoprecipitated with the anti-p62 antibodies (lane b). p62 is indicated by an arrow. (C) A 4 ng sample of TfR mRNA was injected into oocyte nuclei. Oocytes were incubated for 2 minutes before direct cooling to 4°C (a and b) or UV irradiation and transfer at 4°C (c and d). Nuclear extracts were digested with RNases A and T_1 and DNase. Protein components were separated by isoelectrofocusing (IEF) in the first dimension and by SDS-PAGE (10% acrylamide) in the second dimension (SDS). Gels were then either stained with Coomassie Blue (a and c; isoelectric variants of p62 are indicated by arrowheads) or immunoblotted with anti-p62 antibodies 1 (b and d). The crossreactive protein is marked with a star. Bars represent the molecular mass markers of 100.4, 71.7, 43.5 and 29 kDa. The dot corresponds to the pI of *Xenopus* lamins (6.4-6.6) determined by immunoblotting the same membranes with anti-lamin antibodies.

nition particle RNA (He et al., 1994) and the ribosomal subunit (Bataillé et al., 1990) transport by WGA and by the ability of polyspecific anti-nucleoporin antibodies to block the nuclear export of 5 S ribosomal RNA and mature tRNA (Featherstone et al., 1988). However, in contrast to the process of protein import, nucleoporins whose function in RNA export are affected by these reagents have not been identified. At present, we cannot formally exclude the possibility that WGA and nucleoporin antibodies block RNA export through an inhibition of protein import. However, WGA injected into the cytoplasm failed to inhibit the appearence of ribosomal subunits (Bataillé et al., 1990) and newly synthesized RNA (Yoneda et al., 1987) in the cytoplasm, whereas this reagent blocks nuclear import of karyophilic proteins (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988; Wolff et al., 1988). From these data, it appeared essential to identify the targets of WGA-mediated inhibition of mRNA export.

In this report, we were able to detect, by a new approach, RNA-protein complexes formed in vivo along the mRNA export pathway. Our results show that: (i) p62 associates with mRNA; (ii) WGA decreased by 70% the formation of the p62-mRNA complex; (iii) an antibody reacting specifically with

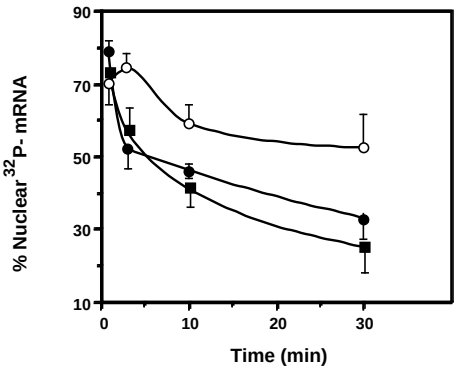

Fig. 5. mRNA export is inhibited by anti-p62 antibodies. A 4 ng sample of ^{32}P-labeled TfR mRNA was injected into oocyte nuclei that had been preinjected 1 hour before with 20 nl of water (■), anti-p62 antibodies (❍, 1 mg/ml of IgG fraction) or anti-p62 antibodies plus recombinant p62 (●, 100 ng/ml). At different times after injection (1, 3, 10 and 30 minutes), oocytes were fixed in 3% acetic acid, 80% ethanol at 4°C and dissected. The radioactivity present in the nucleus and cytoplasm of each single oocyte was determined, and the percentage of radioactivity present in the nucleus calculated. Each point represents the average of three experiments and in each experiment six to eight oocytes were injected per condition.

p62 inhibits mRNA export. Together, these data strongly suggest that p62 is directly involved in mRNA export. This is therefore the first example of a direct interaction between a nucleoporin and a transported molecule. In this report we have focused on one of the major crosslinked product whose binding to mRNA is affected by WGA. However, this does not exclude the possibility that other interactions between glycosylated nucleoporins and RNA that have not been detected so far might occur.

Despite the lack of a known RNA-binding consensus motif within the p62 sequence, it is possible that p62 interacts directly with mRNA. A novel RNA binding domain has recently been described in yeast nucleoporins Nup 145p, 116p and 100 that confers on these proteins RNA-binding activity in vitro (Fabre et al., 1994). Indeed, if interactions between mRNA and nuclear pore proteins are required for mRNA export, they are expected to be transient and most likely to exhibit low affinity. On the other hand, we cannot exclude the possibility that the covalent binding induced by cross-linking between mRNA and the nucleoporin p62 could also occur, due to their proximity in the nuclear pore. Since p62 is localized at both the nucleoplasmic and cytoplasmic peripheries of the nuclear pore complex (Cordes et al., 1991), different possible roles for this protein in mRNA export may be envisaged, such as recognition and/or association of mRNA with the nuclear pore complex as well as in a checkpoint function at the nuclear pore complex exit.

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