A novel function for the 90 kDa heat-shock protein (Hsp90): facilitating nuclear export of 60 S ribosomal subunits

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Ribosomal subunits are assembled in the nucleus, and mature 40 S and 60 S subunits are exported stoichiometrically into the cytoplasm. The nuclear export of ribosomal subunits is a unidirectional, saturable and energy-dependent process. An *in vitro* assay for the nuclear export of 60 S ribosomal subunits involves the use of resealed nuclear envelopes. The export of ribosomal subunits from resealed nuclear envelopes is enhanced by cytoplasmic proteins. Here we present evidence that the export-promoting activity was due to the cytoplasmic 90 kDa heat-shock protein (Hsp90). Isolated, purified Hsp90 vastly enhanced the export of 60 S ribosomal subunits from resealed nuclear envelopes, while inhibition of Hsp90 function, either with the Hsp90-binding drug geldanamycin or with anti-Hsp90 antibodies, resulted in reduced release of 60 S ribosomal subunits. To confirm these findings under *in vivo* conditions, corresponding experiments were performed with *Xenopus* oocytes using microinjection techniques; the results obtained confirmed the findings obtained with resealed nuclear envelopes. These findings suggest that Hsp90 facilitates the nuclear export of 60 S ribosomal subunits, probably by chaperoning protein interactions during the export process.

Key words: geldanamycin, nuclear envelope, nucleocytoplasmic transport, ribosome.

INTRODUCTION

Ribosomal subunits are assembled in the nucleolus from ribosomal proteins and rRNA. In eukaryotic cells, a large ribosomal 45 S precursor rRNA is transcribed and processed to yield 5.8 S, 18 S and 25 S/28 S rRNAs, which are incorporated into ribosomal subunits. Formation of the small 40 S and large 60 S ribosomal subunits requires the nuclear import of approximately 80 ribosomal proteins (reviewed in [1]). The synthesis of ribosomes is dependent on growth rate; the rate of export of ribosomal subunits varies during the cell cycle [2]. Mature 60 S ribosomal subunits have a molecular mass of approx. 2.9 MDa, and are approx. 26 nm in diameter [3,4]. They belong to the largest structures that are transported by the nuclear pore complex (NPC). Permeability measurements with NPCs revealed that they have a passive permeability for molecules up to approx. 5 nm in size ([5] and references therein). It is known that the NPC can undergo large conformational changes during the transport of large cargo substrates [6]. However, compared with other transport processes, much less is known about the export mechanism of ribosomal subunits. Using microinjection techniques in *Xenopus* oocytes it was shown that the nuclear export of mature ribosomal subunits is an active, saturable and unidirectional process. Interestingly, there are opposing results concerning the export of microinjected prokaryotic ribosomal subunits [7,8]. Early studies using *in vitro* measurements of export from isolated nuclei suggested that this process is energy-dependent, temperature-sensitive and requires cytosolic factors [9–11]. Co-injection experiments in *Xenopus* oocytes revealed that the nuclear export of 5 S rRNA, mRNA, tRNA and uracil-rich small nuclear RNA is mediated by independently acting factors. There is evidence, however, that at least the tRNA and 40 S ribosomal subunit export pathways share common, perhaps non-specific (i.e. low-affinity), binding intermediates [12].

In order to investigate the nuclear export of 60 S ribosomal subunits, Hurt et al. [13] developed an assay featuring a green fluorescent protein-tagged variant of the ribosomal protein L25. Another yeast-based system developed by Moy and Silver [14] allows monitoring of the export of the small 40 S ribosomal subunit. The yeast-based *in vivo* assays suggest the need for the Ran cycle and distinct nucleoporins, as well as a subset of transport factors. Recently, a protein was identified as being crucial for the export of the 60 S ribosomal subunit. This protein, Nmd3, assembles with mature 60 S ribosomal subunits and acts as an adaptor for nuclear export via the nuclear export signal receptor exportin [15]. An alternative approach to studying ribosomal nuclear export in eukaryotes was described by Hassel and co-workers [16], who used resealed nuclear envelopes [17,18]. Isolated nuclei can be converted into nuclear envelope vesicles, and during this process can be loaded with distinct export substrates. Release of the included substrates can be analysed under defined conditions [18].

In view of the size of ribosomal subunits, as well as of the extensive exchange of material between nucleus and cytoplasm via the NPC, we asked whether mechanisms exist to support the transport of large structures, such as 60 S ribosomal subunits, through the NPC. To address this issue, the effects of various cytosolic protein fractions on the release of 60 S ribosomal subunits from resealed nuclear envelopes were analysed. The protein responsible for the main export-stimulating effect was identified as the 90 kDa heat-shock protein (Hsp90). Inhibition of Hsp90 function, by the addition of either the Hsp90-specific drug geldanamycin or anti-Hsp90 antibodies, led to a significant decrease in the release of 60 S ribosomal subunits from the

Abbreviations used: NPC, nuclear pore complex; Hsp, heat-shock protein; DTT, dithiothreitol.

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resealed nuclear envelopes. Similar results were obtained using *Xenopus* oocytes and microinjection techniques as an *in vivo* assay. These findings extend the list of cellular processes in which Hsp90 is known to be involved. The possible physiological relevance of this effect is discussed.

EXPERIMENTAL

Preparation of rat liver cytosol and resealed nuclear envelopes

Whole rat liver nuclei were isolated from male Sprague–Dawley rats (150–200 g body weight; Charles River, Sulzfeld, Germany) using the method of Blobel and Potter [19]. Rat liver cytoplasm was prepared from the first supernatant. The supernatant was spun for 1 h at 100000 ***g*** at 4 °C (rotor SW 28; Beckman, Munich, Germany). After removal of floating fat and dialysis against incubation buffer (see below), the cytosol was centrifuged again for another 12 h at 100000 ***g*** at 4 °C in order to remove residual ribosomal subunits and complexes > 30 S. Again, trace levels of floating fat were removed carefully.

The preparation of resealed nuclear envelopes was described in detail previously [17,18]. In brief, nuclei were suspended in TP buffer (10 mM Tris/HCl and 10 mM Na_2HPO_4, pH 8.0) to a DNA concentration of 200 μg/ml, as determined by absorbance at 260 nm. After adding 300 μg of heparin (Liquemin solution; Hoffmann-La Roche Co.; 30 mg/ml) per ml, the suspension was stirred gently for 3 min at room temperature. Vesicles were pelleted at 2000 ***g*** for 2 min at 4 °C and washed twice with STKMC buffer (50 mM Tris/HCl, 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 3.3 mM $CaCl_2$ and 0.5 mM PMSF, pH 7.4). Finally, the vesicles were resuspended in incubation buffer [50 mM Tris/HCl, 25 mM KCl, 2.5 mM $MgCl_2$, 5 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM PMSF, 2 mM dithiothreitol (DTT) and 5 mM spermidine, pH 7.4], adjusted to 5 mg of protein/ml and kept on ice until used. Protein concentrations were determined by the Bio-Rad protein assay using lyophilized BSA (Roth, Karlsruhe, Germany) as standard.

Cytosol fractionation

For cytosol fractionation, a two-pump FPLC system (Amersham Pharmacia, Uppsala, Sweden) was used. On the basis of results of pilot fractionations using Mono Q and Q-Sepharose anion-exchange columns (Amersham Pharmacia), cleared cytosol (see above) was loaded on to a Q-Sepharose column equilibrated with buffer A (20 mM Tris/HCl and 250 mM NaCl, pH 7.4). The column was washed with 20 vol. of buffer A and bound proteins were eluted with a step gradient with buffer B (20 mM Tris/HCl and 450 mM NaCl, pH 7.4). Relevant protein fractions were purified further by gel filtration using a Superose 6 column (Pharmacia Amersham) equilibrated with 75 mM Tris/HCl, 37.5 mM KCl, 7.5 mM NaCl, 3.75 mM $MgCl_2$, 2.5 mM NaH_2PO_4 and 0.25 mM $CaCl_2$, pH 7.4. The resulting fractions containing the enriched protein at high purity were dialysed against incubation buffer for export measurements.

Identification of Hsp90

SDS/PAGE was performed using the procedure described by Laemmli [20] (see below). Proteins intended for N-terminal sequencing were electrotransferred on to a PVDF membrane (Immobilon-P; Millipore) using a tank-blot apparatus (Bio-Rad). Blotting was performed with a buffer containing 11 mM 3-(cyclohexylamino)-1-propanesulphonic acid and 12 % (v/v) methanol, pH 11.0, at a constant 100 mA for 1 h. Proteins were stained with Amino Black 10B (Serva, Heidelberg, Germany). In order to obtain sequence information from the interior of the protein chain, the protein was subjected to cleavage with CNBr. Therefore proteins were re-eluted from the SDS/PAGE matrix using an electroseparation apparatus (Biotrap; Bio-Rad) at a constant 300 V at 4 °C for 5 h. Re-eluted proteins were precipitated using the method described by Wessel and Flügge [21] and incubated in 10 mg/ml CNBr in 70 % formic acid at 4 °C for 24 h. The peptides obtained were separated by HPLC (Jasco, Gross-Umstadt, Germany) using a Brownlee Aquapore 300 C18 column and with a continuous acetonitrile gradient up to 70 % (v/v), containing 0.1 % trifluoroacetic acid.

Preparation of 60 S ribosomal subunits

Ribosomes were prepared from rat liver polysomes using the procedure described by Palmiter [22]. After dissociation of the ribosomes with puromycin (Sigma), separation of the subunits was performed by zonal centrifugation (rotor Ti 15; Beckman) in a sucrose density gradient from 10 % to 40 % (w/v) sucrose, as described [16]. Fractions containing 60 S ribosomal subunits were adjusted to 25 mM $MgCl_2$ and sedimented by centrifugation at 16 °C and 70000 ***g*** for 22 h (rotor SW28; Beckman). Pelleted material was resuspended at 4 °C by slow shaking for 1 h in 50 mM sodium phosphate/5 mM $MgCl_2$, pH 7.4, adjusted to a protein concentration of 12 mg/ml and stored at −80 °C.

Labelling of 60 S ribosomal subunits and Hsp90

The 60 S ribosomal subunits used for export experiments were labelled with ^{125}I (Amersham Pharmacia Biotech) using the chloramine-T method [23]. Free iodine was separated using a Sephadex G25 (Amersham Pharmacia) column equilibrated in 20 mM sodium phosphate, pH 7.5, 10 mM sucrose, 5 mM $MgCl_2$, 2 mM DTT and 0.5 mM PMSF.

For fluorescence microscopy studies, 60 S ribosomal subunits and Hsp90 purified from rat liver were coupled to fluorescent dyes. Coupling of fluorescein-5-maleimide (Pierce, Rockford, IL, U.S.A.) to 60 S ribosomal subunits and of Cy5-maleimide (Molecular Probes Inc., Eugene, OR, U.S.A.) to Hsp90 was carried out according to the manufacturer's instructions. Labelled 60 S ribosomal subunits or labelled Hsp90 were separated from the reaction mixture by gel filtration on a Sephadex G25 column equilibrated in 20 mM sodium phosphate, 10 mM sucrose, 5 mM $MgCl_2$, 2 mM DTT and 0.5 mM PMSF, pH 7.5 (60 S ribosomal subunits), or in 20 mM sodium phosphate, 2 mM DTT and 0.5 mM PMSF, pH 7.4 (Hsp90).

For electron microscopy studies, Hsp90 was biotinylated with 3-(*N*-maleinimidobutyryl)biotin (Serva, Heidelberg, Germany). To 500 μl of a protein solution at 6 mg/ml was added 200 μg of 3-(*N*-maleinimidobutyryl)biotin dissolved in 0.1 M triethanolamine, pH 7.4, followed by stirring for 2 h at room temperature. Free 3-(*N*-maleinimidobutyryl)biotin was separated using a Sephadex G25 column equilibrated in 20 mM sodium phosphate, 2 mM DTT and 0.5 mM PMSF, pH 7.4.

Inclusion of 60 S ribosomal subunits in nuclear envelope vesicles, and measurement of export from resealed nuclear envelopes

Labelled 60 S ribosomal subunits can be included within the nuclear envelopes during preparation of the nuclear envelope vesicles, as described previously [16]. During the incubation step in Ca^{2+}-free, hypo-osmotic buffer (see above), 60 S ribosomal subunits at 50 μg/ml were added to a vesicle concentration of 2 mg/ml. The vesicle suspension was stirred gently for 3 min at room temperature and placed on ice for a further 2 min. Nuclear envelope vesicles were resealed by adding 3 vol. of ice-cold STKMC buffer. Resealed nuclear envelopes, now including 60 S

ribosomal subunits, were harvested by centrifugation (2000 ***g***, 3 min, 4 °C), and washed twice by centrifugation with ice-cold STKMC buffer and with incubation buffer. Inclusion of the 60 S ribosomal subunits was visualized by using fluorescein-labelled 60 S subunits. In the control experiment, fluorescein-labelled 60 S ribosomal subunits were added after the resealing step, stirred gently for 3 min at room temperature, harvested and washed as in the inclusion procedure. Vesicles were observed immediately with a confocal laser scanning microscope without fixation. A Leica CLSM TCS 4D True Confocal Scanner with a Leitz upright DMRBE microscope equipped with 100 × 1.3 numerical aperture Fluotar optics (Leica) was used. Image processing was done on a personal computer using the program SCANware V. 4.2.

^{125}I-labelled 60 S ribosomal subunits were used for export measurements. The procedure used was described in detail previously [16–18]. Reaction mixtures with various compositions were incubated at 37 °C. Export reactions were started by adding the resealed nuclear envelopes containing the 60 S ribosomal subunits. The final concentrations in the incubation mixture were 2 mg/ml vesicle protein and, when used, 1 mg/ml Hsp90, 1.5 mg/ml each of full-length importin β and the truncated importin β-(45–462) fragment, 5 mg/ml each of rat liver cytoplasm and rabbit control serum, and 3 mM ATP. At designated times, 150 μl of the reaction mixture was removed, layered on to two layers of 10 μl of 60 % $HClO_4$ and 40 μl of silicone oil (density 1.03 mg/ml) in Microfuge tubes (Beckman) and centrifuged rapidly in a high-torque Microfuge (Beckman), which reaches a maximum speed after 2 s. After cutting off the tips of the tubes, the radioactivity in the pellet and in the supernatant was measured. The total number of counts always amounted to at least 95 % of the radioactive substrate added.

Cloning and expression of recombinant proteins

Plasmids encoding importin β, a dominant negative importin β-(45–462) fragment and core nucleoplasmin were kindly donated by Dr D. Görlich (ZMBH, Heidelberg, Germany). Core nucleoplasmin is a truncated form of nucleoplasmin lacking the first 149 amino acids bearing the nuclear localization signal [24]. The recombinant proteins have six consecutive histidine residues at their C-terminus, and were affinity purified using Ni^{2+}/nitrilotriacetic acid–Sepharose (Qiagen, Hilden, Germany). Plasmids were transformed into *Escherichia coli* M15/pRep4 (Qiagen) and proteins were expressed as described previously [25,26].

cDNAs for human isoforms of Hsp90α (pUC90α) and Hsp90β (pUC90β) were purchased from StressGen (Victoria, BC, Canada). The coding sequences for Hsp90 proteins were amplified by PCR using Vent DNA polymerase (New England Biolabs, Frankfurt/Main, Germany). The following oligonucleotides were used. Primer set 1: forward, 5′-GCCTAGCCATGGGACCTGAGGAAACCCAGACCCAAG-3′; reverse, 5′-GCGGGATCCGTCTACTTCTTCCATGCGTGATGT-3′; primer set 2: forward, 5′-CATACATGCATGCCTGAGGAAGTGCACCATGGAGAG-3′; reverse, 5′-CAGGAAGATCTATCGACTTCTTCCATGCGAGACGCA-3′. The PCR products obtained with primer sets 1 and 2 were cloned into the *Nco*I/*Bgl*II site of pQE60 and the *Sph*I/*Bgl*II site of pQE70 (Qiagen) respectively, generating vectors pQE60Hsp90α and pQE70Hsp90β. All DNA-modifying enzymes were purchased from New England Biolabs; oligonucleotides were purchased from BioSpring (Frankfurt/Main, Germany). pQE60Hsp90α and pQE70Hsp90β were transformed into *E. coli* SG13009/pRep4 (Qiagen). Cells were grown in 2YT medium [1.6 % (w/v) tryptone, 1 % (w/v) yeast extract and 0.5 % (w/v) NaCl], and protein expression was induced at an A_{600} of 1.5 with 0.1 mM isopropyl β-D-thiogalactoside for 2 h at 30 °C. Cells were harvested and resuspended in 0.2 M Tris, 0.5 M NaCl, 5 mM β-mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin and 40 mg/ml pepstatin, pH 8.0, and lysed with two strokes of a French Press [124 MPa (18000 lb/in^2) cell pressure]. The lysate was cleared by centrifugation for 30 min at 12000 ***g*** and 4 °C. Recombinant Hsp90 proteins were affinity purified via their His_6 tag on an Ni^{2+}/nitrilotriacetic acid Fastflow (Qiagen) FPLC column equilibrated with 10 mM imidazole, 0.5 M NaCl and 50 mM sodium phosphate, pH 8.0. The column was washed with 30 mM imidazole, 0.5 M NaCl and 50 mM sodium phosphate, pH 8.0, and proteins were eluted with a 30–500 mM imidazole gradient. Recombinant Hsp90 proteins were eluted at an imidazole concentration in the range 200–250 mM. Peak fractions were collected and dialysed against 50 mM Tris, 2.5 mM $MgCl_2$, 5 mM NaCl, 2.5 mM Na_2HPO_4 and 25 mM KCl, pH 7.4. Protein concentrations were adjusted to 5 mg/ml using ultrafiltration (see above). Recombinant Hsp90 proteins were purified further using gel filtration on a Superdex 200 FPLC column (Amersham Pharmacia) equilibrated in 50 mM Tris, 2.5 mM $MgCl_2$, 5 mM NaCl, 2.5 mM Na_2HPO_4 and 25 mM KCl, pH 7.4. Fractions containing Hsp90 proteins only were pooled, adjusted to 5 mg/ml (see above) shock-frozen in liquid nitrogen and stored until use at −80 °C.

Gel electrophoresis

SDS/PAGE was performed using the procedure described by Laemmli [20]. For native gel electrophoresis, solutions without SDS or β-mercaptoethanol were used, and no stacking gels were made. BSA and catalase (Combithek; Boehringer Mannheim) were used as marker proteins for native gel electrophoresis. Gels were stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich).

Production of polyclonal antibodies and serum preparation

A New Zealand White rabbit (2.5–3 kg body weight; Charles River) was immunized against Hsp90 purified from rat liver (see above). The immunization procedure was that described by Ausubel et al. [27]. Blood was taken from the marginal ear vein and left to stand for 90 min at room temperature. The clot was removed and the serum was incubated at 4 °C overnight. The remaining red blood cells were removed by centrifugation for 30 min at 1000 ***g*** at 4 °C. Serum was removed and again centrifuged (30 min, 12000 ***g***, 4 °C). Antibodies from the serum of the immunized rabbit were purified with the aid of a Protein G–Sepharose column (Amersham Pharmacia), according to the manufacturer's instructions. Serum derived from a non-immunized rabbit was used as a control. The protein concentration of the control serum was adjusted to 15 mg/ml using ultrafiltration (see above).

Microinjection into *Xenopus* oocytes and export measurements

Defolliculated *Xenopus laevis* oocytes at stages V and VI were kindly provided by Dr G. Schmalzing (University of Frankfurt/Main), and kept in Ori medium (5 mM Hepes/KOH, pH 7.4, 110 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). A volume of 18.4 nl or 50 nl was injected into nuclei or cytosol respectively with the aid of a Bachofer injection apparatus (Bachofer, Reutlingen, Germany). For export measurements, 500 ng of radioactively labelled 60 S ribosomal subunits was injected into each oocyte nucleus. Injected substances were dissolved, unless indicated otherwise, in 20 mM Hepes/KOH,

pH 7.4, 100 mM potassium acetate, 5 mM magnesium acetate and 5 mM sodium acetate. Concentrations of injected substances other than 60 S ribosomal subunits are indicated in the corresponding Figure legends. The anti-Hsp90 antibody employed (N-17) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). An anti-BSA antibody (Sigma-Aldrich) was used for control measurements. To confirm the exact location of the microinjected substance, small amounts of haemoglobin were added to the solutions. At least 10 oocytes at each time point were fixed for 30 min in 1% trichloroacetic acid at 4 °C. Oocytes were dissected manually and radioactivity was determined in the nuclei as well as in the cytosolic fraction.

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal calf serum, 1% (w/v) penicillin, 1% (w/v) streptomycin and 450 mg/l glucose. Cultures were maintained in a humidified incubator (Cytoperm 2; Heraeus) at 5% CO_2 and 37 °C. Cells were removed from plastic dishes by trypsinization and replated on 18 mm × 18 mm glass coverslips. Culture medium was replaced by fresh medium 2–3 h before starting an experiment.

Immunostaining

HeLa cells grown on coverslips were washed three times with PBS (12 mM NaH_2PO_4, 124 mM NaCl and 270 μM KCl, pH 7.4), fixed and permeabilized with 350 mg/ml paraformaldehyde/0.1% Triton X-100 dissolved in PBS. Unspecific binding sites were blocked with 10% (v/v) fetal calf serum in PBS for 30 min at room temperature. For detection of Hsp90, the anti-Hsp90α antibody N-17 (Santa Cruz Biotechnology) was used, at a final concentration of 0.2 μg/ml, dissolved in PBS/1.5% (v/v) fetal calf serum. After incubation with the primary antibody for 60 min at room temperature, the cells were washed three times with PBS. A fluorescein-labelled anti-goat antibody (Santa Cruz) was used as secondary antibody, at a final concentration of 1.5 μg/ml. The incubation procedure was the same as applied for the primary antibody. Mounting on slides was performed as described [28]. Samples were observed with a confocal laser scanning microscope (see above).

Localization of Hsp90 in digitonin-permeabilized HeLa cells

The digitonin-permeabilized cell system as established by Adam et al. [29], essentially as described by Moore and Blobel [28], was used to study the binding of fluorescently labelled Hsp90 to the nuclear envelope. Coverslips containing HeLa cells were washed with 2 × 1 ml of ice-cold 50 mM Hepes/KOH, 110 mM KCl, 5 mM sodium acetate, 5 mM magnesium acetate, 1 mM EGTA, 2 mM DTT and 0.5 mM PMSF, pH 7.3. This buffer was used for all subsequent steps. Permeabilization was performed by incubating the cells for 5 min with 20 μg/ml digitonin. Cells were then washed twice with ice-cold buffer, kept for 10 min on ice for complete cytosol depletion, and again washed twice with ice-cold buffer. Coverslips with cells were laid on 20 μl of buffer containing 100 μg/ml fluorescently labelled Hsp90, 10 mg/ml BSA and 2 mg/ml core nucleoplasmin. BSA and core nucleoplasmin were added to block unspecific binding sites. Cells were incubated for 30 min at room temperature. After washing with buffer, cells were fixed and prepared for microscopy (see above).

Sample preparation for electron microscopy

Nuclei (5 mg/ml) isolated from rat liver were incubated with biotinylated Hsp90 at a final concentration of 2 mg/ml for 30 min at room temperature, and then washed three times with incubation buffer. After washing, the nuclei were fixed for 30 min with 3% paraformaldehyde/0.05% glutaraldehyde dissolved in 0.1 M sodium phosphate, pH 7.4, on ice, and again washed three times with PBS. To block unspecific binding sites and to ensure complete reaction of the fixative agents, nuclei were incubated for 60 min with 2% glycine/1% BSA dissolved in PBS. Biotinylated Hsp90 was detected by the addition of a 1:10 dilution of streptavidin–gold (AuroProbe EM Streptavidin G10; Amersham Pharmacia) in PBS for 60 min at room temperature. Nuclei were washed three times with PBS and again fixed with 3% paraformaldehyde/1% glutaraldehyde dissolved in PBS, followed by three washing steps. For contrast microscopy, the nuclei were incubated for 2 min with 1% osmium tetroxide dissolved in water, and then were washed three times with water followed by increasing concentrations (25%, 50%, 75%, 90%, 95% and 100%) of dimethylformamide. Finally, nuclei were washed with resin LR–gold (Amersham Pharmacia). Polymerization of the resin was started by the addition of 0.6% dibenzoylperoxide. The suspension was mixed gently, and after 3 min the nuclei were pelleted. After 24 h at room temperature the polymerization reaction was complete. Embedded nuclei were sliced with an ultramicrotome (Leitz), to give slices approx. 60 nm thick. After drying, the sections were examined in an electron microscope (Philips EM300).

RESULTS

Export measurements with 60 S ribosomal subunits

Resealed nuclear envelopes serve as a useful method for studying nucleocytoplasmic transport [16–18,30,31]. Fluorescently labelled 60 S ribosomal subunits can be included in nuclear envelopes, and remain stably included after resealing (Figure 1A). Fluorescently labelled 60 S ribosomal subunits are not able to enter nuclear envelope vesicles when incubated with them after the resealing step. Even after high signal amplification, only small amounts of 60 S ribosomal subunits can be detected at the nuclear envelopes (Figure 1C). Ribosomal subunits exported from resealed nuclear envelopes are intact, as judged by sucrose-density analysis [16].

Since all nuclear transport processes are mediated by NPCs (for a review, see [32]), we investigated whether the rate of export of ribosomal subunits can be influenced by blocking the NPCs. A dominant negative importin β fragment, consisting of amino acids 45–462, was shown to inhibit many nuclear transport processes by binding almost irreversibly to components of the NPCs, thus blocking them [26]. Using resealed nuclear envelopes, the efflux of 60 S ribosomal subunits was reduced significantly by the importin β fragment compared with the control reaction. Full-length importin β had no significant effect on the export process (Figure 2). These results indicate that 60 S ribosomal subunits are released from the resealed nuclear envelopes through the NPCs. As shown previously, the passage of cargo through the NPC is not linked directly to energy consumption [33]. Our results with resealed nuclear envelopes are in accordance with these findings. While hydrolysis of nucleoside triphosphates is not required for the translocation of 60 S ribosomal subunits through the NPC, the release of 60 S ribosomal subunits from the resealed nuclear envelopes is dependent on cytosolic factors, and the export-promoting effect of the cytoplasmic extract is greater when ATP is present.

Measurement of the initial export of 60 S ribosomal subunits from resealed nuclear envelopes stimulated by cytoplasmic proteins and ATP gave a value of approx. 5 ng of 60 S ribosomal subunits/μg of vesicle protein (Figure 2). A 1 mg portion of

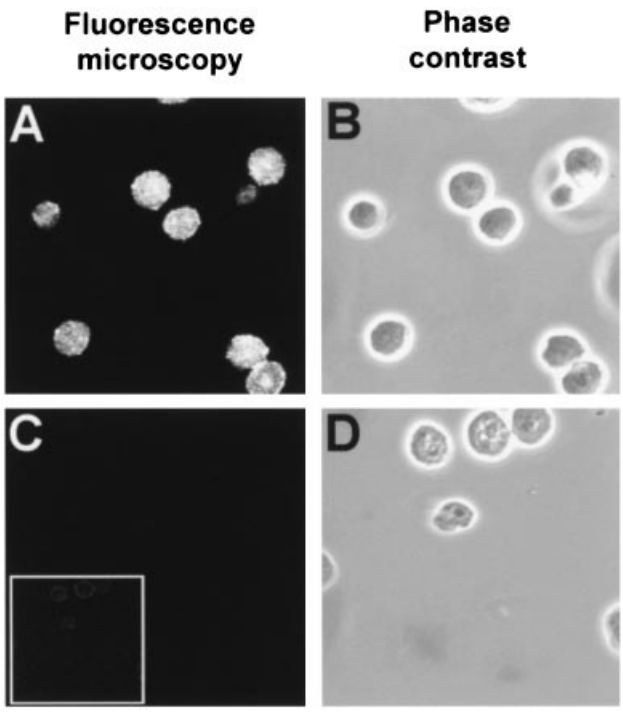

Figure 1 Inclusion of 60 S ribosomal subunits in nuclear envelope vesicles

During smooth permeabilization of freshly prepared rat liver nuclei in a Ca^{2+}-free hypo-osmotic buffer and heparin displacement of chromatin, fluorescein-labelled 60 S ribosomal subunits were included within the nuclear envelope vesicles. On resealing the vesicles in a Ca^{2+}-containing iso-osmotic buffer, the 60 S ribosomal subunits were stably included, and non-included subunits were removed by washing. (**A**) Confocal laser scanning microscopy of nuclear envelope vesicles (NE-vesicles) incubated with 60 S ribosomal subunits (SU) before the resealing step. (**B**) Phase-contrast picture of (**A**). (**C**) As in (**A**), but the nuclear envelope vesicles were incubated with 60 S ribosomal subunits after the resealing step. The inset shows the same picture, but with higher signal amplification. (**D**) Phase-contrast picture of (**C**).

vesicle protein corresponds to approx. 129870 ± 5844 vesicles [31], and liver cells have approx. 4000 NPCs per nucleus [34]. Since one resealed nuclear envelope is derived from one cell nucleus, the amount of NPCs per vesicle is comparable with that per nucleus. Resealed nuclear envelopes retain the NPCs in the correct orientation [17,30]. This means that 1 μg of vesicle protein contains approx. 5.2×10^5 NPCs. Using a molecular mass of 2.9 MDa for the 60 S ribosomal subunit, 5 ng of ribosomal subunits corresponds to 1.7 fmol, or 1.04×10^9 particles. Hence each NPC is capable of exporting approx. 1993 60 S ribosomal subunits per min, or 33.3 60 S ribosomal subunits/s per NPC (see the Discussion).

To compare the results obtained using resealed nuclear envelopes with an *in vivo* system, similar experiments were performed using microinjection techniques in *Xenopus* oocytes. First, the influence of the importin β-(45–462) fragment was analysed by injecting it into either the cytosol or the nuclei of *Xenopus* oocytes. Full-length importin β was again used as a control. Injection of 50 nl of the importin β fragment into the cytosol (calculated final concentration, assuming 1 μl of cytosol per oocyte, of 0.25 mg/ml) had no significant effect on the nuclear export of ribosomal subunits, whereas injection of 18.4 nl of the importin β fragment into the nucleus (calculated final concentration, assuming 100 nl of nucleoplasma, of 3.66 mg/ml) led to a significant decrease of the export rate (Figure 3). Higher concentrations were toxic to the oocytes. Following microinjection of 18.4 nl of full-length importin β into the nuclei (calculated

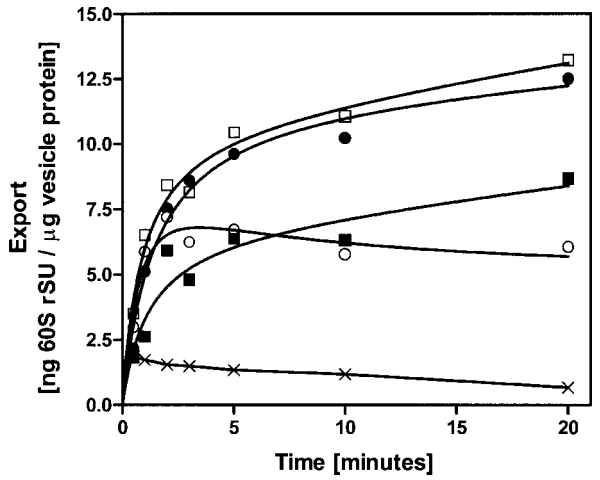

Figure 2 Export of 60 S ribosomal subunits from resealed nuclear envelopes

During preparation of resealed nuclear envelopes from rat liver nuclei, ^{125}I-labelled 60 S ribosomal subunits (rSU) were included in the vesicles. Export reactions were started by adding loaded resealed nuclear envelopes to the reaction mixture. The final concentration of vesicle protein was 2 mg/ml. At the indicated times, aliquots of the reaction mixture were transferred into a Microfuge tube containing 10 μl of perchloric acid and 40 μl of silicone oil, and centrifuged. ×, Control curve at 37 °C in incubation buffer only, without further additions. Importantly, this net efflux was deducted from all subsequent export measurements with resealed nuclear envelopes, including those shown in Figures 5 and 6. Additions were as follows: □, 5 mg/ml rat liver cytosol and 3 mM ATP; ●, 5 mg/ml rat liver cytosol, 3 mM ATP and 1.5 mg/ml full-length importin β; ■, 5 mg/ml rat liver cytosol; ○, 5 mg/ml rat liver cytosol, 3 mM ATP and 1.0 mg/ml importin β-(45–463) fragment.

final concentration 0.8 mg/ml), no changes compared with the control could be observed.

In *Xenopus* oocytes, approx. 60 % of 60 S ribosomal subunits injected into nuclei were exported into the cytoplasm within 6 h under normal (control) conditions (see Figures 3 and 7). Since 500 ng of ribosomal subunits was injected into oocyte nuclei, the export rate was calculated to be of the order of 1.7×10^8 60 S ribosomal subunits/min per oocyte. Assuming a density of

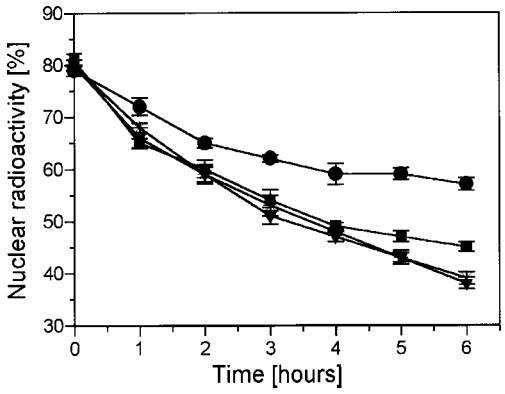

Figure 3 Influence of the importin β-(45–463) fragment on the export of 60 S ribosomal subunits in *Xenopus* oocytes

See the Materials and methods section for experimental details. ▼, Control; ●, co-injection of the importin β-(45–463) fragment into nuclei (final concentration 3.66 mg/ml); ■, co-injection of the importin β-(45–463) fragment into cytoplasm (final concentration 0.25 mg/ml); ×, co-injection of full-length importin β into nuclei (final concentration 0.8 mg/ml).

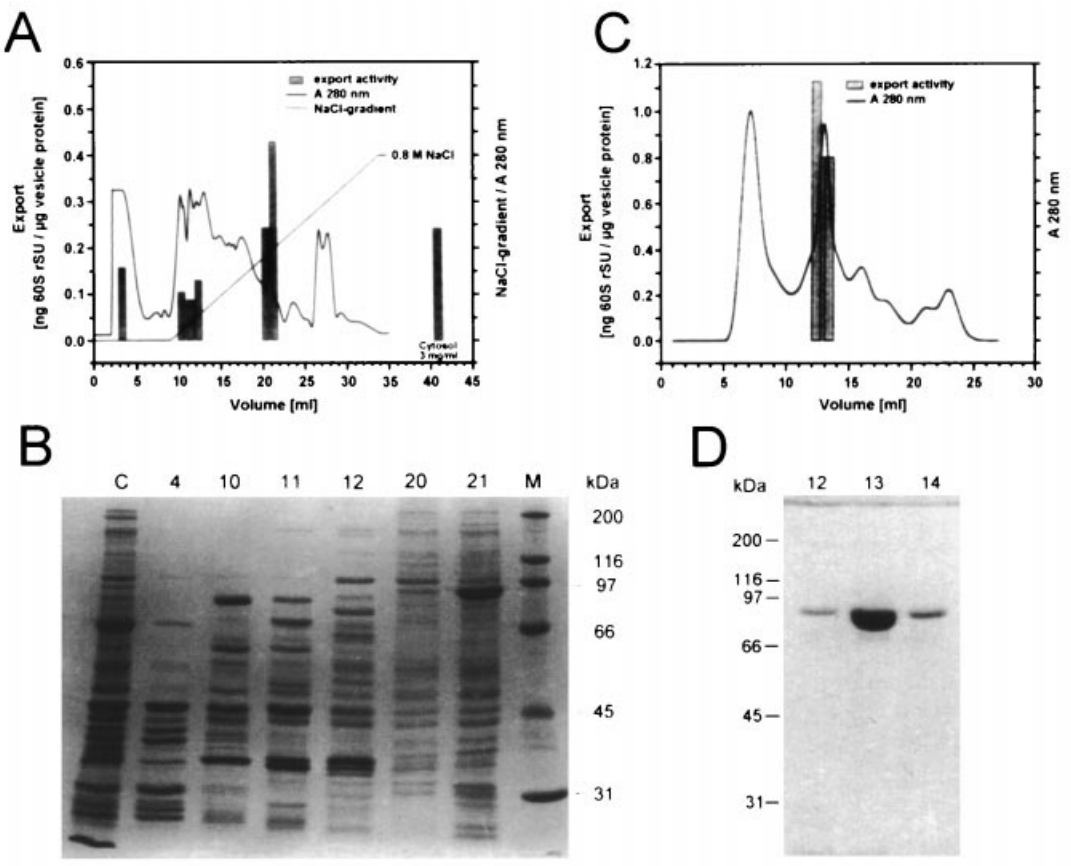

Figure 4 Identification of Hsp90 as an export-stimulating factor

(**A**) Cytosol prepared from rat liver was loaded on to an anion-exchange column and bound proteins were eluted with an NaCl gradient. Protein fractions were dialysed against incubation buffer and tested for stimulation of the export of 60 S ribosomal subunits (rSU) from resealed nuclear envelopes. Resealed nuclear envelopes containing radioactively labelled 60 S ribosomal subunits were incubated with the corresponding protein fractions (final concentration 0.2 mg/ml), and after 10 min the vesicles were pelleted. As a control, the effect of unfractionated cytosol was also tested. (**B**) SDS/PAGE of protein fractions tested for export-stimulating activity as shown in (**A**). C, cytosol, M, marker proteins. (**C**) Further purification by gel filtration of the fraction with the highest export-stimulating activity from the anion-exchange column. Eluted protein fractions were again tested for export-stimulating activity. (**D**). SDS/PAGE of protein fractions obtained after gel filtration.

50 NPCs/μm^2 in *Xenopus* oocytes [35] and an oocyte nucleus diameter of 100 μm, as estimated with the aid of a microscope, the total number of NPCs per oocyte nucleus was calculated to be of the order of 10^7. This gives a value of 17 exported 60 S ribosomal subunits/min per NPC (see the Discussion).

Identification of Hsp90 as a stimulatory factor for the nuclear export of 60 S ribosomal subunits

The presence of a cytosolic factor that stimulates the release of ribosomal ribonucleoprotein particles from isolated rat liver nuclei was proposed by Yu et al. [9], and later studies by this group ascribed the effect to a protein of approx. 70 kDa [10]. In a more recent study, a cytosolic protein fraction was partially purified that also stimulated the release of 60 S ribosomal subunits from resealed nuclear envelopes [16]. In order to identify the protein or proteins responsible for this activity, cytosol prepared from rat liver was fractionated via an anion-exchange column and eluted fractions were analysed for export-stimulating activity. In the protein fraction with the highest export-stimulating activity, a prominent protein with an apparent molecular mass of approx. 95 kDa, as estimated by SDS/PAGE, was visible (Figures 4A and 4B). For large-scale purification of the 95 kDa protein, a Q-Sepharose column was used instead of a Mono Q column for the anion-exchange chromatography step. The protein was enriched further to high purity by a subsequent gel filtration step. This purified protein showed high export-stimulating activity and displayed a prominent band at approx. 95 kDa on SDS/PAGE (Figures 4C and 4D).

To identify this protein, it was electrotransferred on to a PVDF membrane and cleaved with CNBr, and the peptides obtained were subjected to N-terminal degradation. The obtained sequences were PEETQ and MKENQKXIYFIT. A database search using the multiprotein database retrieval system ATLAS via the Munich Information Center for Protein Sequences (MIPS) (http://www.mips.biochem.mpg.de) identified the protein as Hsp90. Mammals have two isoforms of Hsp90, designated Hsp90α and Hsp90β. Indeed, the 95 kDa protein band is a double band (results not shown). The sequences derived from the peptides fit both the Hsp90α and the Hsp90β sequences as deposited in the databases, and hence did not allow us to distinguish between the two isoforms. The proteins in the double band have been purified via a slightly modified version of the preparation scheme and identified by N-terminal sequencing as the Hsp90α and Hsp90β isoforms [36].

Next, native Hsp90 was tested for its ability to enhance the release of 60 S ribosomal subunits from resealed nuclear envelopes. Hsp90 had a much higher export-stimulating activity

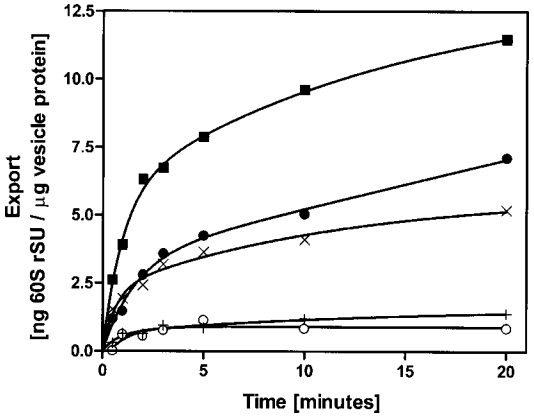

Figure 5 Inhibition by anti-Hsp90 antibodies of the stimulatory effect of Hsp90 on the release of 60 S ribosomal subunits from resealed nuclear envelopes

Additions: ■, 0.5 mg/ml Hsp90 purified from rat liver; ●, 0.5 mg/ml Hsp90 purified from rat liver plus 0.5 mg/ml polyclonal anti-Hsp90 antibodies raised against rat Hsp90 in rabbit; ×, 2 mg/ml rat liver cytoplasm; +, 2 mg/ml serum from non-immunized rabbit; ○, 0.5 mg/ml rabbit anti-(rat Hsp90) antibody. rSU, ribosomal subunits.

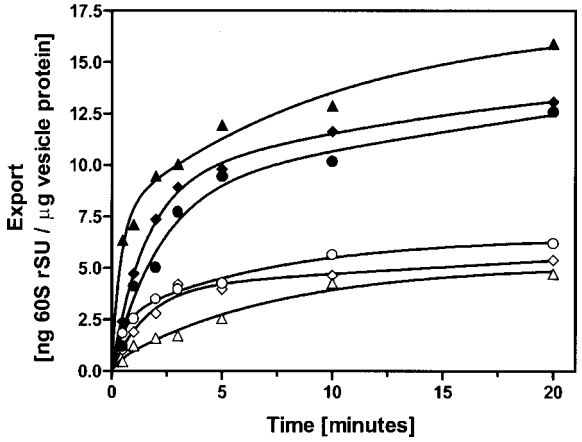

Figure 6 Inhibition by geldanamycin of the stimulatory effect of Hsp90 on the release of 60 S ribosomal subunits from resealed nuclear envelopes

Additions: ▲, 1 mg/ml Hsp90 purified from rat liver; △, 1 mg/ml Hsp90 purified from rat liver plus 100 μM geldanamycin; ●, 1 mg/ml recombinant Hsp90α; ○, 1 mg/ml recombinant Hsp90α plus 100 μM geldanamycin; ◆, 1 mg/ml recombinant Hsp90β; ◇, 1 mg/ml recombinant Hsp90β plus 100 μM geldanamycin. rSU, ribosomal subunits.

compared with the same amount of cytoplasm. The stimulatory effect of Hsp90 on the export of 60 S ribosomal subunits could be inhibited by anti-Hsp90 antibodies. Addition of ATP increased the export-stimulating activity of Hsp90 further (results not shown). As a control, the effect of serum prepared from a non-immunized rabbit was tested for export-stimulating activity, but, as expected, it had no effect in this test system (Figure 5). To further confirm the stimulatory effect of Hsp90 on 60 S ribosomal subunit export, both Hsp90 isoforms were expressed heterologously in *Escherichia coli* and tested for export-stimulating activity. The recombinant expressed Hsp90 proteins also stimulated the release of 60 S ribosomal subunits from resealed nuclear envelopes, albeit with rather lower efficiency. The reason for this is not yet clear. The benzoquinone ansamycin

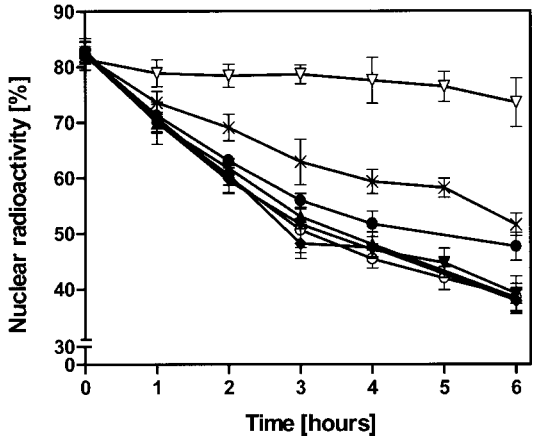

Figure 7 Effects of additional Hsp90 and of inhibition of Hsp90 function in *Xenopus* oocytes on the export of 60 S ribosomal subunits

Conditions: ▽, control (4 °C); ×, injection of a monoclonal anti-Hsp90α antibody into the cytoplasm (final concentration 10 μg/ml); ●, injection of geldanamycin into the cytoplasm (final concentration 0.2 mM); ▼, control (25 °C) with 0.2% DMSO; ○, injection of an anti-BSA antibody into the cytosol (final concentration 10 μg/ml), serving as a control; ◆, injection of Hsp90 purified from rat liver into the cytoplasm (final concentration 50 μg/ml); ▲, co-injection of Hsp90 purified from rat liver into the nucleus (final concentration 0.73 mg/ml). Injection of the anti-Hsp90α antibody, Hsp90 and geldanamycin into the cytoplasm was performed 1 h before the injection of 60 S ribosomal subunits into the nuclei. Hsp90 was co-injected into the nuclei at the same time as the 60 S ribosomal subunits.

antibiotic geldanamycin is a well-known inhibitor of Hsp90, binding tightly within the N-terminal ATP-binding site [37]. Addition of geldanamycin inhibited the export-stimulating activity of all Hsp90 proteins tested (Figure 6). Hsp90 forms dimers via its C-terminal domain [38]. The oligomeric state of the Hsp90 proteins used in our study was checked by native PAGE. Monomeric forms could not be observed, indicating that both Hsp90 isoforms expressed in *E. coli* formed stable dimers (results not shown).

To confirm the results obtained with the resealed nuclear envelopes, the influence of geldanamycin and anti-Hsp90 antibodies, as well as of the further addition of Hsp90, on the export of 60 S ribosomal subunits was analysed in *Xenopus* oocytes. The anti-Hsp90 antibody and geldanamycin were microinjected into the cytoplasm. Geldanamyin was dissolved in DMSO and then diluted to a DMSO concentration of 0.2% (v/v). Both geldanamycin and the anti-Hsp90 antibody clearly reduced the rate of export of 60 S ribosomal subunits from *Xenopus* oocyte nuclei (Figure 7). Higher concentrations of geldanamycin could not be tested because of toxic effects of the DMSO. Further addition of Hsp90, either co-injected into the nuclei or into the cytoplasm, had no effect on the nuclear export of 60 S ribosomal subunits. This may be explained by postulating that the export rate of 60 S ribosomal subunits was already at or near its maximum level.

Interaction of Hsp90 with the nuclear membrane

It seems reasonable to assume that Hsp90 interacts with components of the nuclear envelope in order to exert its export-promoting activity. First, the localization of Hsp90 in HeLa cells was analysed by immunostaining. This approach showed that Hsp90 is distributed widely throughout the cytoplasm, but a distinct association with the nuclear membrane could also be observed (Figure 8A). Many cytosolic proteins that interact with Hsp90 have been characterized. In order to reduce the amount of

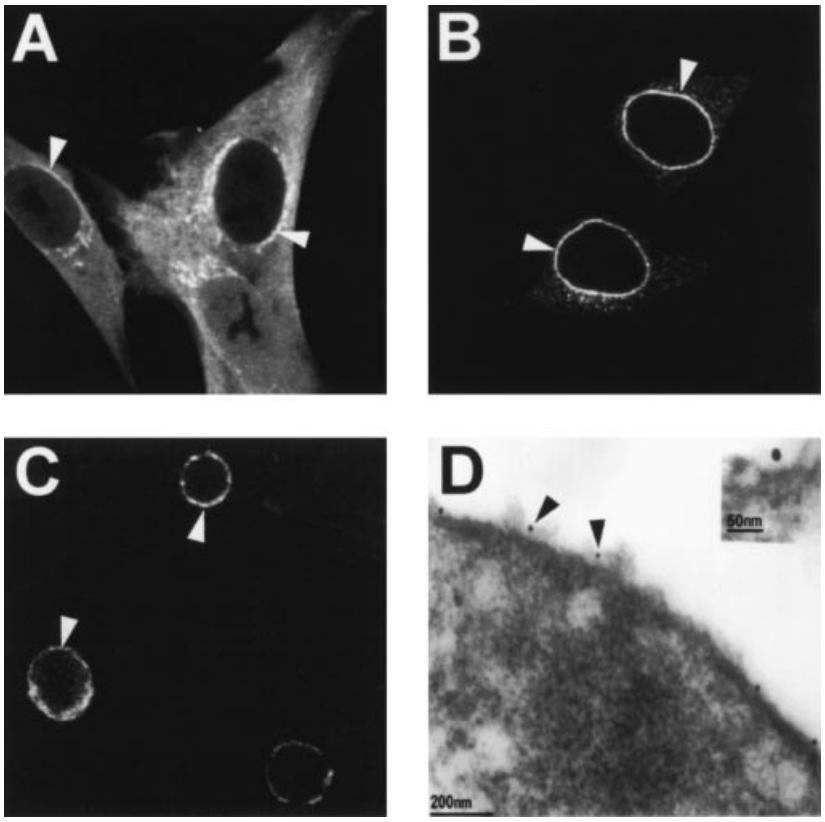

Figure 8 Localization of Hsp90 at the nuclear envelope

(**A**) Distribution of Hsp90 inside HeLa cells, as detected with the aid of an anti-Hsp90α antibody. The primary antibody was detected by a second fluorescently labelled antibody. (**B**) Localization of fluorescein-labelled Hsp90 purified from rat liver in digitonin-permeabilized HeLa cells. Fluorescein-labelled Hsp90 was added to a final concentration of 100 μg/ml in the presence of transport buffer only and incubated for 30 min at room temperature. (**C**) Binding of fluorescein-labelled Hsp90 to resealed nuclear envelopes. Fluorescein-labelled Hsp90 was added at a concentration of 10 μg/ml to a suspension of 2 mg/ml resealed nuclear envelopes and incubated for 15 min at room temperature. Images (**A**)–(**C**) were taken with a confocal laser scanning microscope. Hsp90 localized at the nuclear envelope is indicated by arrowheads. (**D**) Transmission electron microscopy image of biotin-labelled Hsp90 added to cell nuclei isolated from rat liver. Biotin-labelled Hsp90 was detected with gold-labelled streptavidin. Arrowheads indicate Hsp90 localized at the NPCs.

competitive binding partners for Hsp90 in the cytoplasm, the distribution of fluorescently labelled Hsp90 was analysed in digitonin-permeabilized HeLa cells, a test system initially developed to study nuclear transport [29]. Digitonin treatment of the cells permeabilizes the plasma membrane selectively, without impairing the nuclear membrane. During the digitonin treatment, most cytosolic proteins are released [29]. A strong association of fluorescently labelled Hsp90 with the nuclear membrane could be observed in this test system (Figure 8B). Next, the interaction of Hsp90 with resealed nuclear envelopes, which were used as the test system for the export of 60 S ribosomal subunits, was analysed. Again, binding of fluorescently labelled Hsp90 to the nuclear membrane could clearly be observed, but no fluorescence could be detected inside the resealed nuclear envelopes (Figure 8C). Finally, the binding site for Hsp90 at the nuclear membrane was analysed by transmission electron microscopy. This approach revealed Hsp90-binding sites on the outer face of the nuclear membrane, obviously in the area of the NPCs (Figure 8D).

DISCUSSION

Our data for the rate of export of 60 S ribosomal subunits obtained with *Xenopus* oocytes are in good accordance with earlier observations. Bataille et al. [8] observed a maximum export rate for 60 S ribosomal subunits in *Xenopus* oocytes of 1.7×10^7 subunits/min per oocyte. This corresponds to 1.7 60 S ribosomal subunits/min per NPC. Using data from a similar study by Khanna-Gupta and Ware [7], a maximal rate of export of approx. 0.5 ribosomal subunit/min per NPC can be calculated. Görlich and Kutay [39] assumed an export rate of three ribosomal subunits/min per NPC. For the yeast *Saccharomyces cerevisiae* an export rate of approx. 13 subunits/min per NPC can be calculated, assuming 150 NPCs per nucleus [40] and the production of 2000 ribosomes per min [41]. In an earlier study by Wunderlich [42] investigating the export of rRNA from *Tetrahymena* nuclei, the maximal rate of export was calculated to be 45 molecules of both 25 S and 17 S rRNAs/min per NPC. Under *in vivo* conditions, other export processes, as well as import processes, take place. Thus there is a lot of traffic at the NPC, limiting the maximal transport of a specified cargo. With the *in vitro* model of resealed nuclear envelopes in the present study, the observed export velocity was nearly 2000 60 S ribosomal subunits/min per NPC. This is more than 100 times higher than that observed under *in vivo* conditions, implying a high transport capacity of a single NPC. High transport capacities of single NPCs have also been described by Ribbeck and Görlich [43]. Using a test system based on digitonin-permeabilized HeLa cells, they observed that a single NPC can translocate up to 800 molecules of transportin (molecular mass 100 kDa) per s. This

corresponds to a mass exchange of 80 MDa/s for each NPC. A similar value can be calculated from our data for the release of 60 S ribosomal subunits from resealed nuclear envelopes. Expressed in terms of molecular mass, a single NPC allows a mass flow of approx. 100 MDa/s. Ribbeck and Görlich [43] presented a model, dubbed the selective phase hypothesis, to explain such high rates of transport.

The aim of our present study was to identify cytosolic factors that enhance the passage of 60 S ribosomal subunits through NPCs. The export-stimulating effect of cytoplasmic proteins has been reported by various groups [9–11,16]. Recently, Nmd3p was identified as a cytoplasmic protein involved in the export of 60 S ribosomal subunits acting via the nuclear export signal receptor exportin [15]. Gadal and co-workers [44] showed that Nmd3 binds to the ribosomal protein Rpl10p. Using the resealed nuclear envelope *in vitro* test system as a powerful screening model, we identified Hsp90 as an additional cytoplasmic protein that is capable of significantly facilitating the export of 60 S ribosomal subunits. The involvement of Hsp90 in this process was confirmed by corresponding experiments in *Xenopus* oocytes.

Hsp90 is an abundant cytosolic protein that is essential for all eukaryotic cells. Under stress conditions, e.g. heat stress, its synthesis can be enhanced. Under these conditions Hsp90 and other heat-shock proteins protect other proteins from aggregation. The N-terminal region of Hsp90 is an ATP-binding site which also binds the drug geldanamycin, leading to the inhibition of Hsp90. Although the precise role of ATP binding and hydrolysis in Hsp90 function has not yet been elucidated, it has been assumed that the chaperone function of Hsp90 is affected by its ATPase activity (for a recent review, see [45]). Nevertheless, ATP-independent chaperone functions for Hsp90 have been reported [46,47]. In our experiments Hsp90 was able to facilitate the release of 60 S ribosomal subunits from resealed nuclear envelopes without a requirement for ATP, although the addition of ATP enhanced this process. The presence of ATP may enable Hsp90 to work more efficiently, or to mediate energetically unfavourable protein–protein interactions.

Inhibition of Hsp90 function with either antibodies or the drug geldanamycin also reduced the rate of export of 60 S ribosomal subunits from the nucleus under *in vivo* conditions. This supports the conclusion that the export-promoting effect of Hsp90 has physiological relevance. Several proteins interacting with Hsp90 have been described [45]. The mainly cytoplasmic localization of Hsp90 in non-stressed cells is well established (e.g. [48]). In addition, the association of Hsp90 with components of the cytoskeleton has been described (for a review, see [49]). However, the association of Hsp90 with the nuclear membrane seems to occur *in vivo* under certain conditions only ([48]; T. Langer, S. Rosmus and H. Fasold, unpublished work). Our results suggest that Hsp90 has specific binding sites on the nuclear membrane, probably in the area of the NPCs. RNA and ribosomal subunits have also been localized to the above-mentioned cytoskeleton elements (for a review, see [50]). A possible function for Hsp90 as a component of a so-called transportosome, a cellular mechanism promoting transport, especially of steroid hormone receptors, along the cytoskeleton has been discussed [51]. The connection of the NPCs with cytoskeletal elements is well established [52]. As demonstrated by Kiseleva et al. [6], the passage of large cargoes across the NPCs requires large conformational changes in both the NPC and the cargo. It is conceivable that Hsp90 supports these structural changes. Perez-Terzic et al. [53] found that nuclear export is favoured over nuclear import under conditions of cellular hypertrophy. In hypertrophic cells, e.g. hypertrophied cardiac myocytes, the amount of mRNA being transported into the cytosol for protein synthesis is vastly increased [54]. Since ribosomes are also required for protein synthesis, an enhanced synthesis of ribosomes under these conditions can also be assumed. Interestingly, cardiotrophin-1, a factor capable of inducing cardiac myocyte hypertrophy [55], also enhances the synthesis of Hsp70 and Hsp90 [56]. Our results suggest that the enhanced synthesis of Hsp90 in hypertrophic cells is likely to enable the cells to maintain a frictionless nuclear export of large cargo. For the nuclear export of 60 S ribosomal subunits, the following model can be proposed. Passage of 60 S ribosomal subunits through the NPC, requiring maximum dilatation of the NPC, is facilitated by Hsp90 due to its chaperone function. Subsequently, Hsp90 allows transfer of the 60 S ribosomal subunits from the NPC to the cytoskeleton, followed by chaperonage of the 60 S ribosomal subunits through the cell along cytoskeletal transport routes to their final destination.

In view of the selective phase hypothesis [43], which assumes a hydrophobic meshwork inside the NPC composed of Phe-rich nucleoporins, Hsp90 may chaperone the transit of 60 S ribosomal subunits through the NPC by promoting interactions between the hydrophobic meshwork and hydrophobic regions of the transport cargo, which are normally not exposed to the surface. Thus Hsp90 may help to dissolve the cargo in the hydrophobic environment of the NPC.

It was demonstrated previously that heat shock leads to the formation of ribosome crystals inside the nucleus [57]. It can be envisaged that heat shock results in much of the Hsp90 being used to prevent protein aggregation, so that there is not enough 'free' Hsp90 remaining to exert its chaperone function for transport processes across the nuclear membrane. Our findings, in addition to previous studies, supplement the recent identification of Nmd3p involvement in the export of 60 S ribosomal subunits. While Nmd3p may indeed bind to the 60 S ribosomal subunit in order to condition it for subsequent linkage to the NPC, the actual passage of the 60 S ribosomal subunit through the NPC might be mediated and catalysed by Hsp90. In view of the enormous size of 60 S ribosomal subunits, it seems reasonable that their export is mediated through a more complex transport mechanism, probably involving more factors as well as direct interactions of the 60 S ribosomal subunits with the NPC, than export processes for small transport cargoes such as proteins and tRNA. In conclusion, we now have substantial evidence that Hsp90 is involved directly in nuclear transport processes, at least in those of large cargoes such as 60 S ribosomal subunits.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 474 Intrazelluläre Transport und Regulationsvorgänge). We are indebted to Dr Gerd Multhaup (Labor für Molekulare Neuropatholgie, ZMBH, Heidelberg, Germany) for amino acid sequencing. Geldanamycin was kindly provided by the NCI Experimental Drug Division, NIH, Bethesda, MD, U.S.A.

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Received 11 September 2001/20 November 2001; accepted 13 December 2001