Antibodies against 70-kD Heat Shock Cognate Protein Inhibit Mediated Nuclear Import of Karyophilic Proteins

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Abstract. Previously, we found that anti-DDDED antibodies strongly inhibited in vivo nuclear transport of nuclear proteins and that these antibodies recognized a protein of 69 kD (p69) from rat liver nuclear envelopes that showed specific binding activities to the nuclear location sequences (NLSs) of nucleoplasmin and SV-40 large T-antigen. Here we identified this protein as the 70-kD heat shock cognate protein (hsc70) based on its mass, isoelectric point, cellular localization, and partial amino acid sequences. Competition studies indicated that the recombinant hsc70 expressed in *Escherichia coli* binds to transport competent SV-40 T-antigen NLS more strongly than to the point mutated transport incompetent mutant NLS. To investigate the possible involvement of hsc70 in nuclear transport, we examined the effect of anti-hsc70 rabbit antibodies on the nuclear accumulation of karyophilic proteins. When injected into the cytoplasm of tissue culture cells, anti-hsc70 strongly inhibited the nuclear import of nucleoplasmin, SV-40 T-antigen NLS bearing BSA and histone H1. In contrast, anti-hsc70 IgG did not prevent the diffusion of lysozyme or 17.4-kD FITC-dextran into the nuclei. After injection of these antibodies, cells continued RNA synthesis and were viable. These results indicate that hsc70 interacts with NLS-containing proteins in the cytoplasm before their nuclear import.

THE nuclear import of karyophilic proteins is a selective, mediated process. Proteins enter the nucleus through the nuclear pore complex, a large proteinaceous structure present in the nuclear envelope (Feldherr et al., 1984). The pore complex contains an aqueous channel of approximately 100-Å diam that allows the non-selective passive diffusion of microinjected molecules smaller than ~40 kD (for reviews see Dingwall and Laskey, 1986; Newport and Forbes, 1987; Gerace and Burke, 1988). But much larger karyophilic proteins, such as RNP (500 kD) and signal-containing immunoglobulin M (900 kD) can also be efficiently transported into the nucleus through nuclear pores (Michaud and Goldfarb, 1992; Yoneda et al., 1992), apparently because the pore channel expands to allow translocation of karyophilic proteins (Akey and Goldfarb, 1989).

Nuclear transport involves at least two steps, binding to the cytoplasmic face of nuclear pores, and subsequent translocation through the nuclear pore complex. The latter step requires ATP (Richardson et al., 1988; Newmeyer and Forbes, 1988), and is inhibited by wheat germ agglutinin (WGA)[1] or antibodies that recognize nucleoporins, a family of nuclear pore proteins that contain unique O-linked *N*-acetylglucosamine (GlcNAc) residues (Finlay et al., 1987; Yoneda et al., 1987; Featherstone et al., 1988). In vitro studies have indicated that nuclear transport requires multiple cytoplasmic factors (Newmeyer and Forbes, 1990; Adam and Gerace, 1991; Sterne-Marr et al., 1992; Moore and Blobel, 1992) as well as pore complex proteins (Finlay and Forbes, 1990). Recently, Moore and Blobel (1992) demonstrated that the binding and translocation steps are mediated by distinct cytoplasmic fractions.

The selective nuclear import of proteins is directed by short amino acid sequences termed nuclear location signal sequences (NLSs) (for recent reviews see Silver, 1991; Garcia-Bustos et al., 1991). Characteristically, NLSs contain a high proportion of basic amino acids, which are essential for their function. However, there seems to be no strong consensus sequence among the various NLSs identified, although the amino acid sequence of each NLS is specific and critical for its function. For example, point mutations of key basic residues such as ^{128}Lys of SV-40 T-NLS dramatically reduce its nuclear targeting activity (Lanford and Butel, 1984; Kalderon et al., 1984; Lanford et al., 1988).

Saturation kinetics provided an early indication that nuclear import is a receptor-mediated process (Goldfarb et al., 1986). Many attempts have been made to identify NLS receptors. Candidate NLS-binding proteins have been identified by cross-linking (Adam et al., 1989; Yamasaki et

1. *Abbreviations used in this paper*: GlcNAc, *N*-acetylglucosamine; hsc70, 70-kD heat-shocked cognate protein; hsps, heat shock proteins; HTP, hydroxylapatite; IPTG, isopropyl-β-D-thiogalactopyranoside; KLH, keyhole limpet hemocyanin; NLSs, nuclear location signal sequences; SMPB, succinimidyl 4-(*p*-maleimidophenyl) butyrate; WGA, wheat germ agglutinin.

al., 1989; Li and Thomas, 1989; Benditt et al., 1989) or ligand blotting (Silver et al., 1989; Meier and Blobel, 1990; Lee et al., 1991). Some of these proteins were found in the cytoplasm (100- and 70-kD proteins; Yamasaki et al., 1989), some in the nucleus (66-kD protein; Li and Thomas, 1989) (76- 67-, 59-, and 58-kD proteins; Benditt et al., 1989), and some in both these fractions (59- and 69-kD proteins; Adam et al., 1989) (140- and 55-kD protiens; Yamasaki et al., 1989) of mammalian cells.

Breeuwer and Goldfarb (1990) physiologically demonstrated the presence of a saturable NLS-binding protein(s) in the cytoplasm of cultured mammalian cells. Adam and Gerace (1991) purified NEM-sensitive cytosolic NLS-binding proteins of 54 and 56 kD identified by chemical cross-linking with SV-40 T-NLS as a probe, and showed that these proteins support nuclear import in a cell-free transport system using semi-permeabilized cultured mammalian cells. Stochaj et al. (1991) purified yeast NLS-binding protein of 70 kD identified by ligand blotting using SV-40 T-NLS and other nuclear proteins as probes. Antibodies against this protein cross-react with cytoplasmic 70-kD proteins from various cell sources including vertebrate cells, and inhibit nuclear protein import in semi-permeabilized cells (Stochaj and Silver, 1992). These proteins may be cytoplasmic receptors of NLS-containing proteins in nuclear transport.

We attempted to identify NLS-binding proteins with antibodies raised against synthetic DDDED peptide. Our strategy was based on the idea that a stretch of acidic amino acids in the NLS receptor may interact electrostatically with the very basic amino acids of T-antigen–like NLSs (PKKKRKV). The cytoplasmic injection of anti-DDDED antibodies strongly inhibited the in vivo nuclear transport of various nuclear proteins (Yoneda et al., 1988). Anti-DDDED antibodies recognized several proteins in nuclear fractions. Among these proteins, we focused on a 69-kD nuclear envelope protein (p69) that bound tightly to nucleoplasmin-Sepharose, and was specifically eluted by peptides based on the NLSs of nucleoplasmin and SV-40 T-antigen (Imamoto-Sonobe et al., 1990).

In this study, we purified p69 and found that this protein is the 70-kD heat shock cognate protein (hsc70). Furthermore, we found that antibodies against hsc70 strongly inhibited the nuclear imports of karyophilic proteins when injected into the cytoplasm of cultured mammalian cells. This inhibition was specific to the mediated import of karyophiles because these antibodies did not inhibit the diffusion of small non-karyophilic proteins into the nucleus. After injection of these antibodies, cells were viable and continued RNA synthesis, indicating that the inhibition was not the result of pleiotropic physiological effects.

Hsc70 is known as the uncoating ATPase for clathrin-coated vesicles (Chappell et al., 1986). The heat shock family of proteins (hsps) behave as "chaperones," and have been implicated in promoting protein folding, transport, and assembly in both eucaryotic and procaryotic systems (for reviews see Lindquist and Craig, 1988; Ellis and van der Vies, 1991; Gething and Sambrook, 1992). Translocation of nascent proteins into the ER and mitochondria requires constitutively expressed members of the hsp70 family on both sides of the membrane (Chirico et al., 1988; Deshaies et al., 1988; Vogel et al., 1990; Scherer et al., 1990; Kang et al., 1990). Their action is considered to facilitate membrane translocation by binding hydrophobic polypeptide domains following the leader sequences of unfolded nascent polypeptides until they can be unloaded at a specific site for transport or assembly. The characteristics of nuclear transport are due to a unique transport process through the large aqueous channels of nuclear pores. The findings that SV-40 T-antigen NLS can target even 260 Å colloidal gold particles (Feldherr et al., 1984; Dworetzky et al., 1988), and that mAbs against nuclear proteins can accumulate in the nucleus by binding to their antigens in vivo (Tsuneoka et al., 1986; Borer et al., 1989) suggest that karyophilic proteins are not unfolded appreciably during their translocation across the nuclear envelope.

Hsc70 itself is known to be localized in both the cytoplasm and the nucleus, and to shuttle between the nucleus and the cytoplasm (Mandell and Feldherr, 1990). In this paper we demonstrate the involvement of hsc70 in nuclear transport in living cells and discuss its role in nuclear transport in connection with its NLS-binding activity.

Materials and Methods

Cell Culture and Cell Labeling

HEL-R66, an established cell line of human embryonic lung cells was cultured, plated for microinjection experiments, and labeled with [^{3}H]uridine as described previously (Yoneda et al., 1987). HeLa cells were cultured in MEM supplemented with 10% FBS. For labeling, HeLa cells were cultured (1.5×10^6 cells/10-cm tissue culture plate) for 18 h in MEM containing 1.5 mg/l of methionine, 10% FCS and [^{35}S]methionine (12 MBq/1.5×10^6 cells).

Preparation of Peptides, Nuclear Targeting Proteins, and Affinity Gel

Peptides were synthesized with a model 430A synthesizer (Applied Biosystems, Inc., Foster City, CA), and purified by HPLC as described previously (Imamoto-Sonobe et al., 1990). Histone H1 was purified from calf thymus as described previously (Sanders, 1977). Nucleoplasmin was purified from *Xenopus laevis* oocytes as described previously (Imamoto-Sonobe et al., 1990). BSA was chemically conjugated to synthetic T-peptide (CPKKKRKVEDP) by use of succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB) as described previously (Yoneda et al., 1988). Nucleoplasmin and hsc70 were conjugated to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) at a protein concentration of 1 mg/ml.

Partial Purification of p69 from Bovine Liver Nuclear Envelopes

p69 was partially purified from high-salt detergent extracts of bovine liver nuclear envelopes, the equivalent fraction of rat liver nuclei in which we originally detected p69, and the presence of p69 was determined by NLS-binding assay on nucleoplasmin-Sepharose as previously described (Imamoto-Sonobe et al., 1990). Nuclei were isolated from freshly excised bovine liver as described by Imamoto-Sonobe et al. (1988), and crude nuclear envelopes were isolated using DNase I by the method described by Dwyer and Blobel (1976). One unit of nuclear envelope was defined as that derived from one OD_{260} of nuclei. Based on the method described by Snow et al. (1987), crude nuclear envelopes were treated sequentially with high-salt buffer (0.5 M NaCl, 0.1 mM $MgCl_2$, 10% sucrose, 10 mM triethanolamine, pH 7.5, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin) and low-salt detergent buffer (2% NP-40, 5 mM $MgCl_2$, 10% sucrose, 10 mM triethanolamine, pH 7.5, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin), at an envelope concentration of 250 U/ml. The final precipitate was extracted with high-salt detergent buffer (20 mM MES, pH 6.0, 1.14% Triton X-114, 0.3 M KCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 10 μg/ml leupeptin), at an envelope concentration of 2,000 U/ml, and the extract was used as starting material for purification of p69. It was incubated at 37°C for 3 min and then centrifuged to obtain a Triton X-114 non-partitioned supernatant

(Goding, 1984). Extract equivalent to 10,000 U of nuclear envelopes was applied to 2 ml of hydroxylapatite gel (HTP; Bio Rad Laboratories, Cambridge, MA) equilibrated with a solution of 20 mM potassium phosphate, pH 7.2, 0.3 M NaCl, 1 mM DTT, 0.2 mM PMSF, and 10 μg/ml leupeptin, and developed with a linear gradient of 20–250 mM potassium phosphate. The p69 recovered in the first 1/3 of the fractions was purified further in a linear gradient of 3–25% (wt/vol) sucrose (40,000 rpm, 12h) (model SW41Ti; Beckman Instruments, Palo Alto, CA) in a solution of 10 mM triethanolamine, pH 7.5, 0.1 mM $MgCl_2$, 0.3 M NaCl, 1 mM DTT, 0.2 mM PMSF, and 10 μg/ml leupeptin.

Purification of p69 (Hsc70) from the Cytoplasm of Ehrlich Ascites Tumor Cells

Freshly harvested Ehrlich ascites tumor cells from the abdominal cavity of mice were washed with PBS and lysed in hypotonic buffer A (20 mM Hepes, pH 7.2, 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml cytochalasin B) by 10 strokes of a homogenizer. The lysate was clarified by centrifugation at 45,000 rpm for 90 min (model 50.2Ti; Beckman Instruments) and applied to DEAE-Sephacel equilibrated with buffer B (20 mM Hepes, pH 7.2, 30 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin). Materials were eluted with buffer B containing 130 mM KCl. The fraction containing p69 was applied to HTP gel equilibrated with buffer B. The column was washed with buffer C (30 mM potassium phosphate, pH 7.2, 0.3 M KCl, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin), and then p69 was eluted with buffer C containing 80 mM potassium phosphate, pH 7.2 (buffer D), and applied to a phenyl-Sepharose column equilibrated with buffer D. The flow through fractions were pooled, supplemented with ATP at a final concentration of 1 mM, and applied to a column of chelating-Cellulofine (charged with copper ions) equilibrated with buffer E (50 mM potassium phosphate, pH 7.2, 0.5 M KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mM PMSF 10 μg/ml leupeptin). p69 was then eluted in a linear gradient from buffer E to buffer F (50 mM Tris-acetate, pH 3.0, 0.5 M KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.2 mM PMSF, 10 μg/ml leupeptin). Eluted fractions were neutralized with 1.5 M Tris-HCl, pH 8.8, and then dialyzed against buffer B. ATP was added to p69 immediately after its recovery from phenyl-Sepharose, and chelating-Cellulofine chromatography was performed in the presence of 1 mM ATP because addition of ATP increased the recovery of p69. About 1 mg of p69 protein was recovered from 30 ml of cell pellet. The optimal elution conditions of p69 on each chromatography were determined by immunoblotting with anti-p69 mouse serum as a probe.

Purification of Recombinant Hsc70

The human hsc70 full-length cDNA clone (pHSC7) was isolated from a lamda ZAP cDNA library constructed from poly(A)$^+$ RNA of HeLa cells using ^{32}P-labeled oligonucleotide corresponding to the 5'-end coding sequence of human hsc70 cDNA as a probe. A bacterial expression plasmid (pETHsc70) was constructed by inserting human hsc70 cDNA between the NdeI and BamHI sites of the expression vector pET3c. An NdeI site containing the AUG initiation codon of the hsc70 cDNA was generated using a synthetic oligonucleotide to create the same coding sequence as that of the original human hsc70 cDNA. *Escherichia coli* cells transformed with pETHsc70 were grown in Super Broth medium containing ampicillin at 37°C to an OD_{550}=0.5. Expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for 1.5 h at 37°C. Hsc70 was purified from inclusion bodies, which contained nearly 80% of the expressed protein. Cells were harvested by centrifugation and were resuspended at 2/100 ml of the original culture volume in buffer G (50 mM Tris-HCl, pH 8.3, 100 mM NaCl, 1 mM EDTA). Lysozyme was added at 1 mg/ml, and the cell suspension was incubated with occasional gentle shaking for 30 min on ice. Then, the suspension was adjusted to 10% of the original volume of culture by adding buffer G containing 5 mM DTT and 1 mM PMSF, and the cells were ruptured by sonication. Inclusion bodies were collected by centrifugation (20 min at 50,000 *g*, 4°C), and washed by sonication and centrifugation in buffer G. Finally, the pellet was resuspended in buffer H (6 M guanidinium-HCl, 10 mM DTT, 20 mM Tris-HCl, pH 7.4), and proteins were extracted. The extract was cleared by centrifugation (20 min at 10,000 *g*, 4°C) and dialyzed against buffer I (20 mM Hepes, pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT). Aggregates that formed during dialysis were removed by centrifugation, bearing most of the hsc70 in the supernatant, which was then applied to ATP-agarose (linked through C-8; Sigma Chemical Co., St. Louis, MO) column. The column was washed successively with buffer I, buffer I containing 0.5 M NaCl, and buffer I. Hsc70 was then eluted with buffer I containing 3 mM ATP (Schlossman et al., 1984). The final preparation contained hsc70 protein of more than 95% purity (determined by SDS-PAGE). The purified recombinant hsc70 supported nuclear transport in semi-permeabilized cells with the same efficiency as that of hsc70 purified from Ehrlich ascites tumor cell cytoplasm (unpublished observation).

Fractionation of Cells

Freshly harvested Ehrlich ascites tumor cells were lysed in hypotonic buffer A as described above. The cell lysates were centrifuged (10,000 rpm, 15 min, in an Eppendorf tube; Brinkman Instruments Inc., Westbury, NY) to yield a supernatant (cytoplasm) and a precipitate. Nuclei were purified from the precipitate by centrifugation through a sucrose cushion as described by Blobel and Potter (1966). Nuclei treated with DNase I (50 μg/ml, room temperature, 20 min) in buffer J (10% sucrose, 10 mM triethanolamine, pH 7.5, 0.1 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin) were centrifuged to obtain a supernatant (DNase I extract) and precipitate of crude nuclear envelopes. Proteins were extracted from the nuclear envelopes sequentially with the following solutions: high-salt buffer (10% sucrose, 20 mM MES, pH 6.0, 0.5 M NaCl, 0.1 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin), low-salt detergent buffer (buffer J containing 2% Triton X-100 and 5 mM $MgCl_2$) and high-salt detergent buffer (20 mM MES, pH 6.0, 2% Triton X-100, 0.3 M NaCl, 1 mM EDTA, 0.2 mM PMSF, and 10 μg/ml leupeptin).

For the results in Fig. 7, crude nuclear envelopes were isolated from HeLa cells as described above and nuclear envelope proteins were extracted directly with modified high-salt detergent buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 2% NP-40, 5 mM $MgCl_2$, 0.2 mM PMSF, 10 μg/ml leupeptin).

Antibodies

For immunizations, materials were injected into animals first with complete Freund's adjuvant, and subsequently with incomplete Freund's adjuvant. Anti-p69 mouse serum was prepared by intraperitoneal injections of partially purified bovine liver nuclear envelope p69 separated on SDS-PAGE gel and located by staining with 0.25% Coomassie brilliant blue (1 μg protein/each immunization). Rabbit anti-hsc70 antibodies were prepared by subcutaneous injection of hsc70 purified from Ehrlich ascites tumor cell cytoplasm (40 μg protein/immunization) as described above and antibodies specific for hsc70 were isolated by affinity chromatography on purified hsc70-conjugated Sepharose 4B. For microinjection experiments, affinity-purified antibodies were concentrated in a Centricon 30 (Amicon Corp., Danvers, MA). mAb Q1 was prepared using a synthetic peptide CGGDDD-EDGG (DDDED-peptide) chemically conjugated to keyhole limpet hemocyanin (KLH) by use of SMPB for immunizations, as previously described (Yoneda et al., 1988). Spleen cells from an immunized BALB/c mouse (by intraperitoneal injections, except for a final boost by intravenous injection; 100 μg protein/injection) were fused with myeloma cells (SP2/0) using polyethylene glycol-4,000. Culture supernatants were screened by antibody capture assay in polyvinylchloride plates coated with DDDED-peptide–conjugated ovalbumin and ^{125}I-anti-mouse IgG (heavy and light chain specific) was used for detections. Hybridomas that reacted with KLH cross-linked only with SMPB were neglected. Of the mAbs against DDDED-peptide, the antibody named Q1 reacted with hsc70 as shown by the radioimmunoassay described above in a hsc70-coated plate. Q1 antibody (IgM) was purified from ascitic fluid of mice with implanted Q1 hybridomas by precipitation with 40% saturation of ammonium sulfate followed by Sephacryl S-400 chromatography on a column equilibrated with 20 mM borate, pH 8.0, 0.5 M NaCl. Mouse mAb C8 (IgM), which did not interact specifically with cellular proteins (determined by indirect immunofluorescence and immunoprecipitation) was used as a control.

NLS-binding Analysis

NLS-binding activities were determined on nucleoplasmin-Sepharose as described previously (Imamoto-Sonobe et al., 1990). For analysis of the NLS binding activity of the high-salt detergent extracts of nuclear envelopes (see Figs. 1 *c*, and 5, *c* and *d*), the sample was diluted to 200 U/ml with the incubation buffer K(50 mM Hepes, pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 15 mM octylglucoside, 0.5 mM DTT, 10 μg/ml leupeptin, 5 μg/ml pepstatin) before incubation with nucleoplasmin-Sepharose. After incubation (1 h at 4°C), the Sepharose was washed with incubation buffer K, and eluted sequentially with incubation buffer K containing 1 mM mutant T-peptide (CPKTKRKVEDP) and buffer K containing 1 mM T-peptide (CPKKKR-KVEDP). Samples were analyzed by SDS-PAGE followed by immunoblotting probed with anti-p69 mouse serum and anti-hsc70 rabbit antibodies.

For results in Fig. 6, chromatographically purified recombinant hsc70 was labeled with ^{125}I (3×10^6 cpm/μg sp act protein) with enzymobeads (Bio-Rad), and then incubated with nucleoplasmin-Sepharose in incubation buffer K containing 1 mg/ml BSA and 0.25 mM ATP (6 h at 4°C), in the presence of various amounts of wild type $T_{(24)}$-peptide containing a flanking sequence (CYDDEATADAQHAAPPKKKRKVED) or its point mutated mutant-$T_{(24)}$ peptide (CYDDEATADAQHAAPPKNKRKVED). The gel was washed with incubation buffer K containing 0.5 mg/ml ovalbumin and 0.25 mM ATP, and then incubated in the same incubation buffer containing 2 mM nucleoplasmin-peptide (YAVKRPAATKKAGQAKKKKLDC; nucleoplasmin NLS) at 4°C for 12 h. Proteins eluted from the gel were analyzed by SDS-PAGE followed by autoradiography.

Sequence Analysis

p69 purified from the cytoplasm of Ehrlich ascites tumor cells (~2 nmoles) was digested with *Achromobacter* protease I (lysylendopeptidase; Wako Pure Chemicals Industries, Ltd., Chū oku, Osaka) at an enzyme to substrate ratio of 1:100 (mole/mole) in 1 ml of 10 mM Tris-HCl, pH 9.5, at 37°C for 6 h (Tsunasawa et al., 1989). The resulting peptides were separated by HPLC on a μBondasphere column (3.9 × 15 cm, C_4, 300 Å; Millipore Corporation, Walters Chromatography, Milford, Massachusetts) with a linear gradient of 0-60% 2-propanol/acetonitrile (7/3, vol/vol) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min for 60 min. The sequences of five isolated peptides were determined in a gas-phase sequenator (model 477A; Applied Biosystems).

Immunoblottings

Proteins were separated by 10% SDS-PAGE or two-dimensional gel electrophoresis and transferred electrophoretically to nitrocellulose sheets. Western blots were probed with anti-p69 mouse serum (1/300 dilution) or affinity-purified anti-hsc70 rabbit antibodies (5 μg/ml; the specificities of antibodies were confirmed by raising the IgG concentration to 200 μg/ml and showing that no additional protein bands or spots were detected) in buffer containing 20 mM Tris-HCl, pH 7.5, 1% skim milk, and 0.3 M NaCl for 4 h at room temperature after blocking with PBS containing 3% skim milk for 2 h. Mouse and rabbit antibodies were detected with alkaline phosphatase–conjugated goat antibodies to mouse or rabbit IgG (Bio-Rad) by the standard method.

Immunoprecipitations

HeLa cell nuclear envelope proteins (see above) or ^{125}I-hsc70 (described above) was incubated with 25 μg of mouse mAb in 300 μl of a solution of 50 mM Tris-HCl, pH 7.5, 2% NP-40, 0.5 M NaCl, 5 mM $MgCl_2$, 5 mg/ml BSA, 0.2 mM PMSF, and 10 μg/ml leupeptin at 4°C for 1 h. Proteins bound to Q1 were precipitated with rabbit anti–mouse IgM and protein A-Sepharose by incubation for another 1 h after addition of these proteins to the extracts.

Two-dimensional Gel Electrophoresis

This procedure was carried out as described previously (Yoneda et al., 1985).

Results

Identification of Nuclear Envelope 69kD Protein Showing NLS-binding Activity with Hsc70

A rat liver nuclear envelope protein of 69 kD (p69) recognized by anti-DDDED antibodies bound tightly to nucleoplasmin-Sepharose, and was specifically eluted by peptides based on the NLSs of both SV-40 large T-antigen and nucleoplasmin (Imamoto-Sonobe et al., 1990). The 69-kD protein in the high-salt detergent extract from bovine liver nuclear envelopes, the equivalent fraction of rat liver nuclei in which we detected p69 (Imamoto-Sonobe et al., 1990), showed the same NLS-binding activity. We partially purified p69 from bovine liver nuclear envelopes (Fig. 1 *a*), and raised mouse antibodies against this protein (Fig. 1 *b*). As shown in Fig. 1 *c*, the 69-kD protein detected by the mouse serum in the nuclear envelope extracts was eluted specifically from nucleoplasmin-Sepharose with 1.0 mM T-peptide (transport competent) but only slightly with 1.0 mM mutant T-peptide (transport incompetent). The specific NLS-binding activity of the partially purified 69-kD protein shows that this protein is the bovine homologue of rat p69 (Imamoto-Sonobe et al., 1990).

On immunoblotting, this mouse antiserum recognized a protein of 69 kD not only in nuclear envelope fractions but also in cytoplasmic fractions of bovine, mouse, and human cells (data not shown). The protein band of ~45 kD recognized by the mouse antiserum (see Fig. 1, *b* and *c*) was often not detected in cultured cell extracts and its intensity varied in different experiments.

We found that the 69-kD protein was more abundant in rapidly growing cells than in non-dividing cells such as those of liver tissue. Cell fractionation studies showed that most of the 69-kD protein was extracted in the cytoplasmic fraction with hypotonic buffer (Fig. 2). Therefore, using anti-p69 mouse serum as probe, we purified the 69-kD protein from the cytoplasmic fraction of Ehrlich ascites tumor cells extracted with hypotonic buffer (Fig. 3 *a*). The final preparation migrated as a single component of pI 5.8 on two-dimensional gel electrophoresis (Fig. 3 *b*). As shown in Fig. 4, the amino acid sequences of five peptides derived from this protein were found to be identical to those of rat 70-kD heat shock cognate protein (hsc70) reported previously (O'Malley et al., 1985). From its mass, isoelectric point, cellular localization, and partial amino acid sequences, we concluded that the 69-kD protein recognized by anti-p69 mouse serum is hsc70.

In further studies, rabbits were immunized with purified hsc70, and antibodies specific to hsc70 were affinity purified with purified hsc70-conjugated Sepharose. Affinity-purified anti–hsc70 antibodies recognized a single protein band of 69 kD in both the cytoplasmic and nuclear envelope fractions on Western blots (Fig. 5 *a*). The cytoplasmic and nuclear envelope 69-kD proteins recognized by these antibodies migrated as a single component of pI 5.8 on two dimensional gel electrophoresis (Fig. 5 *b*).

To confirm that the nuclear envelope-associated 69-kD protein (p69) that showed NLS-binding activity was hsc70, we examined whether highly specific anti-hsc70 antibodies could actually recognize p69. As shown in Fig. 5 *c*, these antibodies recognized the nuclear envelope 69-kD protein that bound tightly to nucleoplasmin-Sepharose and was specifically eluted with 1.0 mM T-peptide (but only slightly with the same concentration of mutant T-peptide). The protein eluted with peptide detected by these antibodies migrated as a single component of pI 5.8 on two-dimensional gel electrophoresis (Fig. 5 *d*). Therefore, we concluded that p69, the nuclear envelope 69-kD protein that showed specific NLS-binding activity, is hsc70.

Interaction of Recombinant Hsc70 Expressed in E. Coli with NLS

We examined the interactions of purified hsc70 with NLS. For this, recombinant hsc70 expressed in *E. coli* was purified by chromatography, iodinated, and incubated with nucleoplasmin-Sepharose in the presence of various amounts of transport competent synthetic SV-40 T-NLS or transport in-

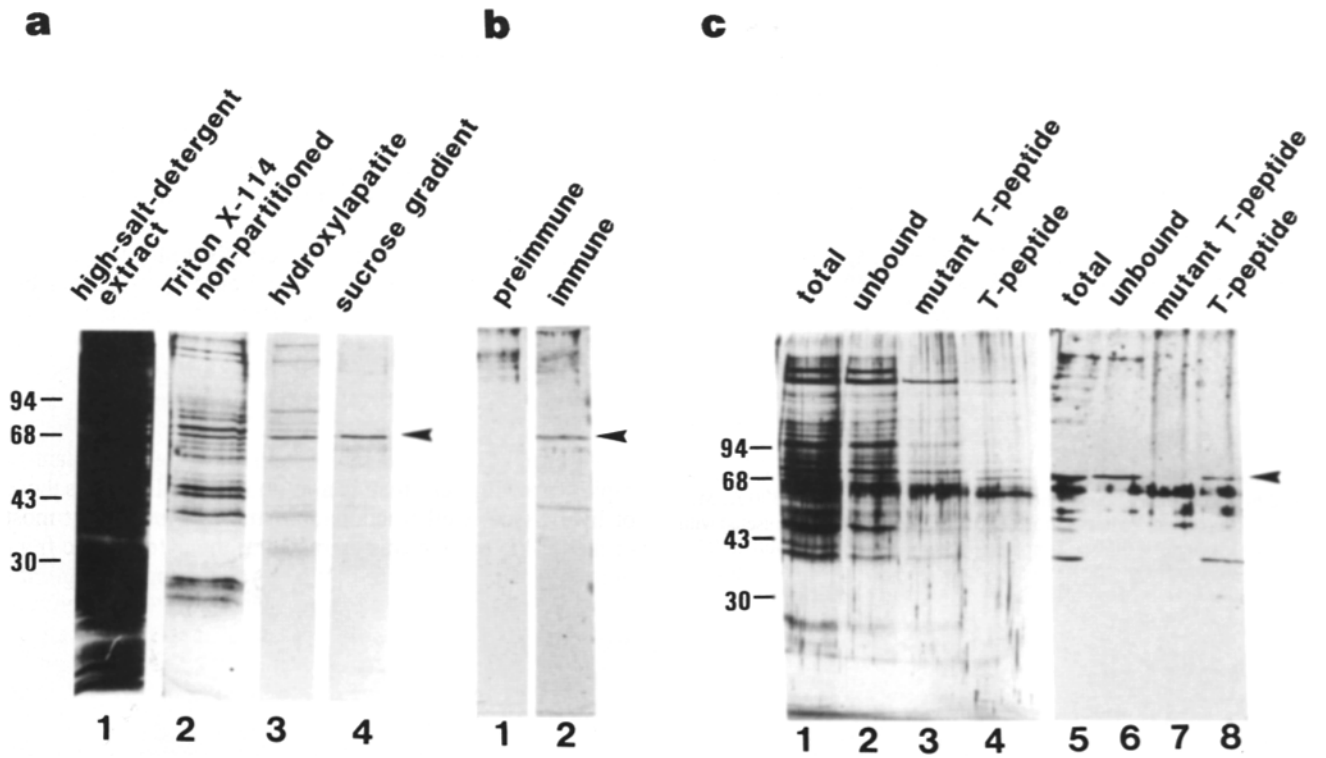


Figure 1. Partial purification of p69 from nuclear envelopes. (*a*) Silver stained profile of proteins at each purification step separated by SDS-PAGE. (Lane *1*) High-salt detergent extract of bovine liver nuclear envelopes; (lane *2*) Triton X-114 non-partitioned fractions; (lane *3*) hydroxylapatite fraction; (lane *4*) sucrose gradient fraction. Proteins equivalent to 10 U of nuclear envelopes were loaded in each lane. (*b*) Western blots tested with anti-p69 mouse antibodies raised against partially purified p69 excised from gel. Lanes *1* and *2* are Western blots of partially purified p69 (lane *4* in Fig. 1 *a*) incubated with preimmune serum (lane *1*) and anti-p69 mouse serum (lane *2*). (*c*) NLS binding assay of 69-kD protein detected with anti-p69 mouse serum. The Triton X-114 non-partitioned fraction (lane *2* in Fig. 1 *a*) equivalent to 250 U of nuclear envelopes was mixed with nucleoplasmin-Sepharose (100-μl gel). Incubation and elution of protein from nucleoplasmin-Sepharose with synthetic peptides were carried out as described under Materials and Methods. Lanes *1–4* show the profiles of proteins separated by SDS-PAGE and stained with silver, and lanes *5–8* are Western blots of samples corresponding to lanes *1–4* incubated with anti-p69 mouse serum. (Lanes *1* and *5*) Total proteins mixed with nucleoplasmin-Sepharose; (lanes *2* and *6*) proteins not bound to nucleoplasmin-Sepharose; (lanes *3* and *7*) proteins eluted with 1 mM mutant T-peptide; (lanes *4* and *8*) proteins eluted with 1 mM T-peptide. The molecular weight markers were phosphorylase b, 94 kD; BSA, 68 kD; ovalbumin, 43 kD; and carbonic anhydrase, 30 kD. Arrowheads indicate the position of p69.

competent point mutated mutant NLS (see Materials and Methods). Hsc70 that specifically bound to nucleoplasmin-NLS was eluted by incubating the Sepharose with an excess amount of nucleoplasmin-peptide (Imamoto-Sonobe et al., 1990). As shown in Fig. 6, the NLS-specific binding of hsc70 to nucleoplasmin-Sepharose was effectively blocked by wild type NLS, while mutant NLS was only slightly inhibitory. These findings are consistent with previous results (Imamoto-Sonobe et al., 1990), and indicate that under our experimental conditions, hsc70 shows specific NLS-binding activity.

Recognition of Hsc70 by Antibody Raised against DDDED Peptide

To reassess the original identification of p69 by anti-DDDED polyclonal antibodies, we obtained mAbs against the DDDED peptide. One of these, Q1 (IgM), precipitated a single protein band of 69 kD from a crude nuclear envelope extract of [^{35}S]methionine labeled HeLa cells (Fig. 7 *a*). Anti-hsc70 rabbit antibodies recognized this immunoprecipitated protein band on an immunoblot (Fig. 7 *b*). Q1 antibody also reacted with purified hsc70 coated on polyvinylchloride plates (see Materials and Methods). Moreover, Q1 antibody specifically precipitated iodinated hsc70, whereas control mouse monoclonal antibody C8 (IgM) did not (Fig. 7 *c*). These results show that antibody against the DDDED peptide recognizes hsc70.

Inhibition by Anti-hsc70 Antibodies of Nuclear Import of Karyophilic Proteins

We examined the effects of affinity purified anti-hsc70 antibodies on the in vivo nuclear transport of karyophilic proteins. For this, affinity purified anti-hsc70 antibodies were injected with nucleoplasmin, or SV-40 T-antigen NLS bearing BSA (T-BSA) into the cytoplasm of cultured human embryonic lung cells (HEL). After incubation at 37°C for 30 min, the cells were fixed and the localizations of injected proteins were examined by fluorescence microscopy (Yoneda et al., 1988). As shown in Fig. 8, anti-hsc70 antibodies inhibited nuclear transport of karyophilic proteins. In typical cases, injection of a solution of anti-hsc70 antibodies at a

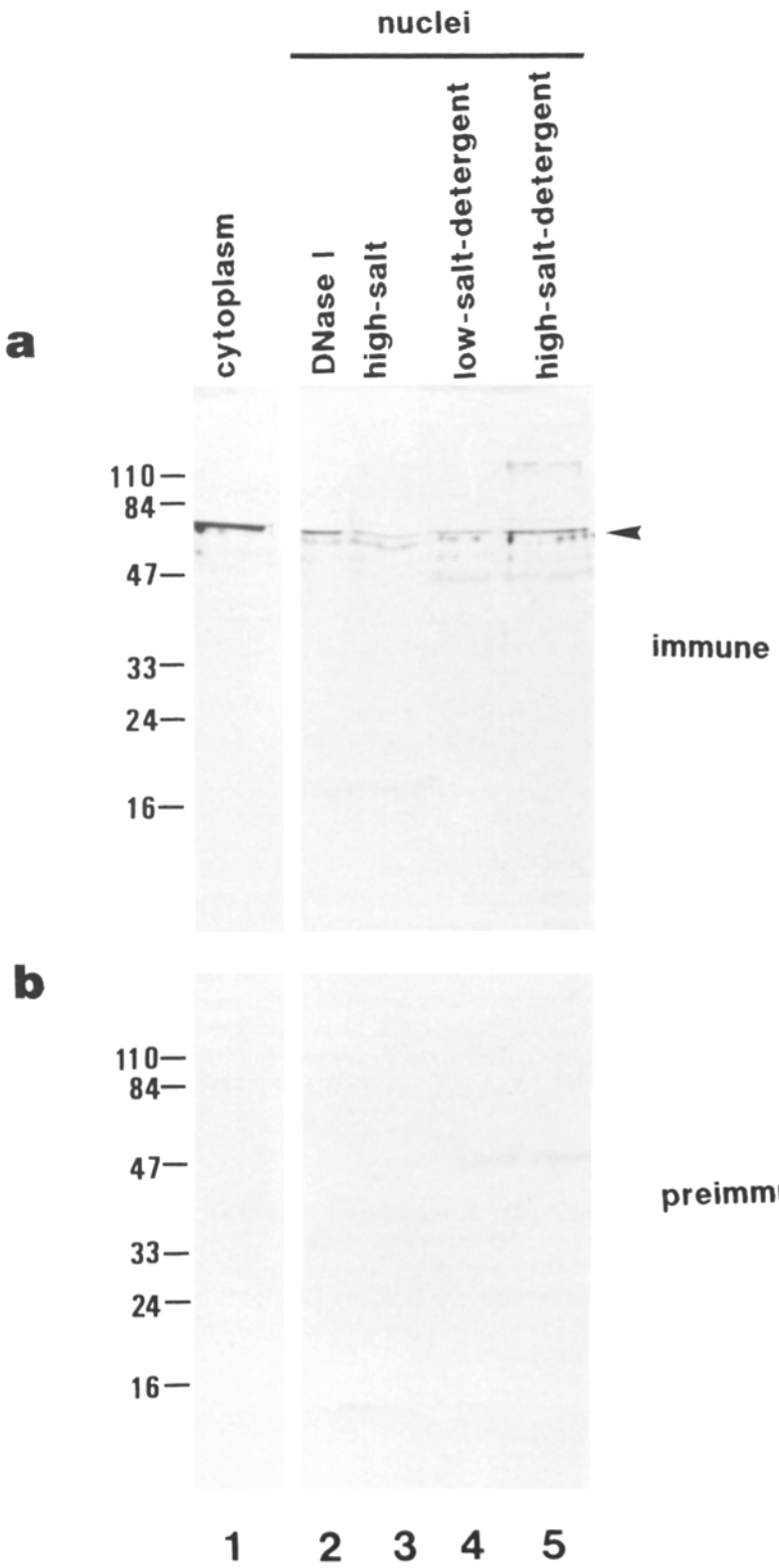


Figure 2. Cellular localization of p69. Western blots of cytoplasmic and nuclear fractions from Ehrlich ascites tumor cells incubated with anti-p69 mouse serum (*a*) and preimmune serum (*b*). Samples of 20 μg of proteins were loaded in the lanes. Results are for cytoplasmic proteins (lane *1*) and proteins sequentially extracted from nuclei with DNase I (lane *2*), high-salt (lane *3*), low-salt detergent (lane *4*), and high-salt detergent (lane *5*). The molecular weight markers were phosphorylase b, 110 kD; BSA, 84 kD; ovalbumin, 47 kD; carbonic anhydrase, 33 kD; soybean trypsin inhibitor, 24 kD; and lysozyme, 16 kD (Bio-Rad's prestained standards). The arrowhead indicates the position of p69.

protein concentration of 20 mg/ml completely inhibited the nuclear transports of both nucleoplasmin and T-BSA (Fig. 8, *d* and *h*). The concentration of anti-hsc70 antibodies required for complete inhibition varied with the lot of affinity purified antibodies (from 10 to 30 mg/ml). As shown in Fig. 8 *j*, after injection of these antibodies at lower protein concentration, small amounts of nucleoplasmin (and also T-BSA) migrated into the nucleus, indicating that small proportions of the nuclear proteins escaped from inhibition and entered the nucleus. The transport inhibitory activity of these antibodies was blocked by chromatographically purified bacterially expressed recombinant hsc70, confirming that the inhibitory

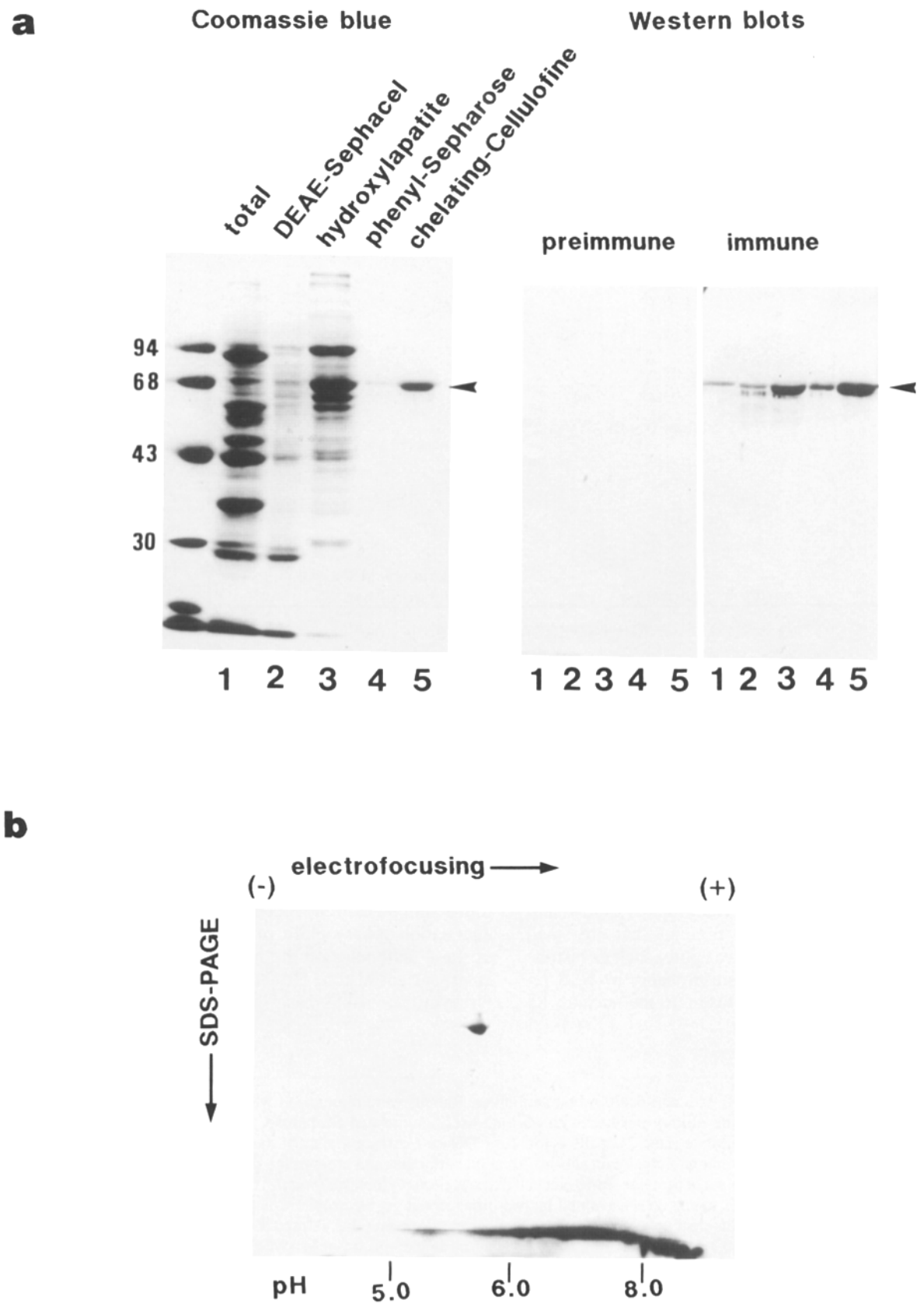
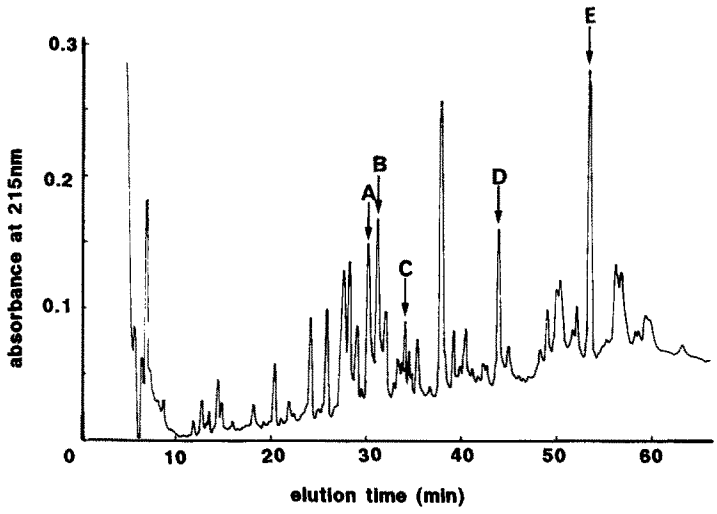


Figure 3. Purification of p69 from Ehrlich ascites tumor cell cytoplasm. (*a*) Coomassie blue profile of proteins from pooled fractions at each purification step separated by SDS-PAGE and Western blots of the corresponding proteins incubated with anti-p69 mouse serum and preimmune serum. (Lane *1*) Total cytosol (30 μg); (lane *2*) DEAE-Sephacel fraction (10 μg); (lane *3*) hydroxylapatite fraction (20 μg); (lane *4*) phenyl-Sepharose fraction (2 μg); (lane 5) chelating-Cellulofine fraction (2 μg). (*b*) Coomassie blue profile of purified p69 (10 μg) separated by two-dimensional gel electrophoresis. Molecular weight markers (left-most lane) were as for Fig. 1. Arrowheads indicate the position of p69.

a



b

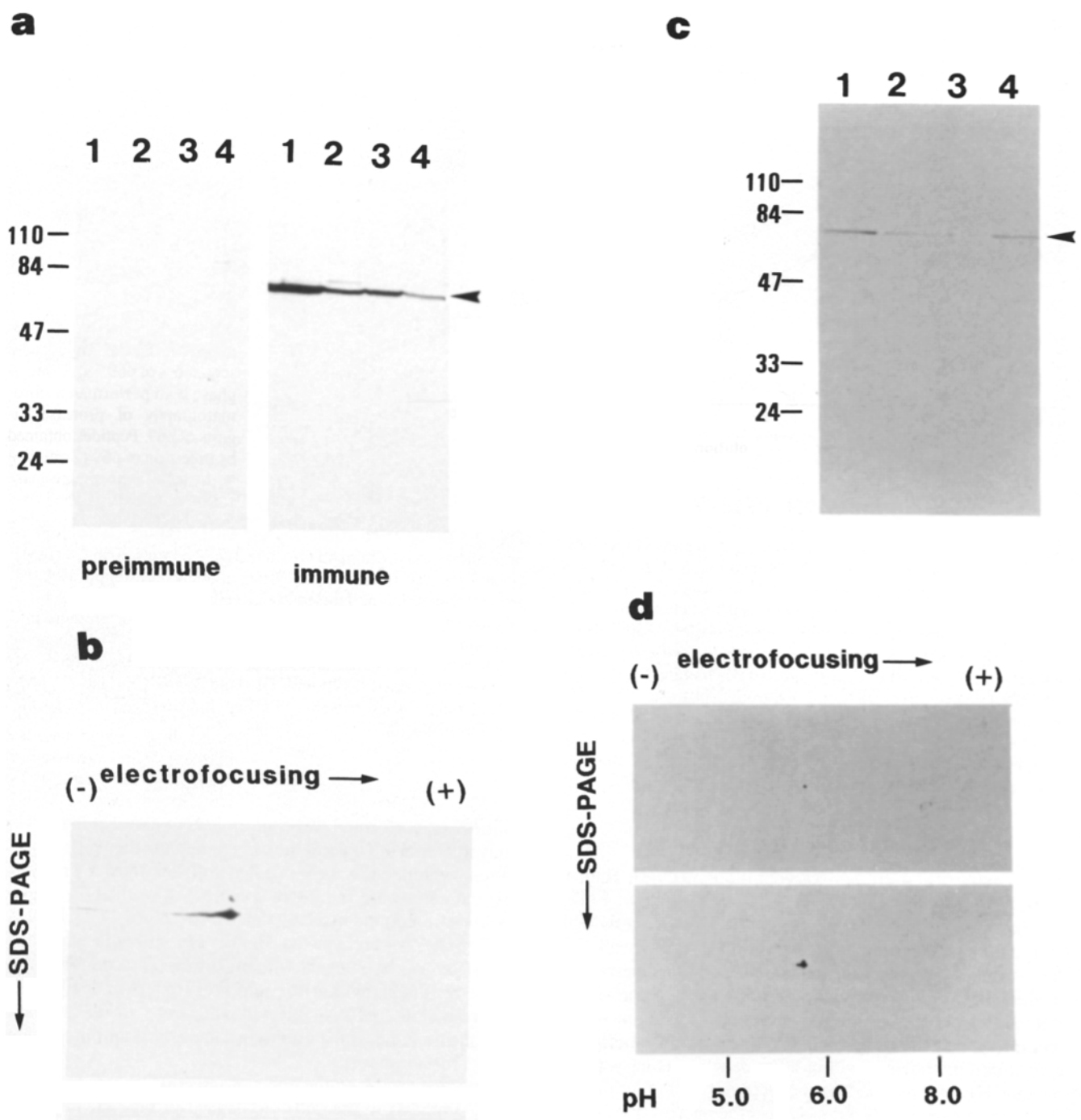
Peptide	Amino acid sequence	Residue No. in the primary structure of hsc 70
A	LYQSAGGMPGGMPGGFPGGGAPPSGGASS	610 – 638
B	HWPFMVVNDAGRPK	89 – 102
C	NEIISWLDK	575 – 583
D	DAGTIAGLNVLRIINEPTAAAIAYGLDK	160 – 187
E	RTLSSSTQASI	272 – 282

Figure 4. Partial amino acid sequences of p69. (*a*) Reverse-phase high performance chromatography of protease digests of p69. Peptides obtained by digestion of p69 (2 nmoles) with *Achromobacter* protease I were chromatographed on a μBondasphere column (3.9 × 15 cm, C_4, 300 Å; (Millipore Corporation) as described under Materials and Methods. The peptides in the five peaks indicated by arrows (*A–E*) were sequenced by Edman degradation. (*b*) Amino acid sequences of the five peptides (*A–E*) indicated in *a*. The corresponding residue numbers in the primary structure of hsc70 (O'Malley et al., 1985) are also indicated.

effect was not caused by contaminating antibodies or proteins (Fig. 9).

Breeuwer and Goldfarb (1990) reported that small non-karyophilic proteins, such as lysozyme, rapidly diffuse into the nucleus, whereas karyophilic proteins that are small enough to diffuse through the nuclear pore, such as histone H1, are complexed in the cytoplasm probably by NLS receptor(s), and that they do not diffuse into the nucleus. So small karyophilic proteins such as histone H1 (21 kD) must accumulate in the nucleus by a receptor mediated import pathway. To determine whether the inhibitory effects of anti-hsc70 antibodies were specific to the mediated nuclear accumulation of karyophilic proteins, we examined the effects of these antibodies on the nuclear imports of small molecules, such as histone H1 (21-kD basic karyophilic protein), lysozyme (14.4-kD basic non-karyophilic protein), and

Figure 5. Identification of p69 as Hsc70 with anti-hsc70 rabbit antibodies. Rabbits were immunized with purified hsc70 described in Fig. 3, and antibodies specific to hsc70 were affinity purified with purified hsc70-conjugated Sepharose. (*a*) Proteins from the supernatant (100,000 *g*, 60 min) (lane *1*) and precipitate (lane *2*) of the cytoplasm; DNase I extracts (lane *3*); nuclear envelope precipitate (lane *4*) from Ehrlich ascites tumor cells were immunoblotted with anti-hsc70 rabbit antibodies and preimmune serum. Samples of 30 μg of proteins were loaded in each lane. (*b*) Western blots of proteins from total cell extracts (*top*), cytoplasm (*middle*) (lane *1* in *a*), and nuclear envelopes (*bottom*) (lane *4* in *a*) of Ehrlich ascites tumor cells separated by two-dimensional gel electrophoresis probed with anti-hsc70 antibodies. (*c*) NLS-binding assay of hsc70 in the nuclear envelope fraction. Nuclear envelope proteins extracted with high-salt detergent buffer (20 mM MES, pH 6.0, 2% Triton X-100, 0.3 M NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 0.2 mM PMSF) from Ehrlich ascites tumor cell nuclear envelopes were mixed with nucleoplasmin-Sepharose as in Fig. 1 *c*. Incubation and elution of protein from nucleoplasmin-Sepharose with synthetic peptides were carried out as described under Materials and Methods. Total proteins mixed with nucleoplasmin-Sepharose (lane *1*), proteins not bound to nucleoplasmin-Sepharose (lane *2*), proteins eluted with 1 mM mutant T-peptide (lane *3*), and proteins eluted with 1 mM T-peptide (lane *4*) were analyzed by SDS-PAGE followed by immunoblotting probed with anti-hsc70 rabbit antibodies. (*d*) Materials specifically eluted with 1.0 mM T-peptide from nucleoplasmin-Sepharose (proteins corresponding to Fig. 5 *c*, lane *4*) alone (*top*), or the same materials mixed with purified hsc70 (*bottom*) were separated by two-dimensional gel electrophoresis, and immunoblotted with anti-hsc70 antibodies. The material examined migrated as a single protein spot of 69 kD, pI 5.8, and co-migrated with purified hsc70. The molecular weight markers were as for Fig. 2. Arrowheads indicated the position of hsc70.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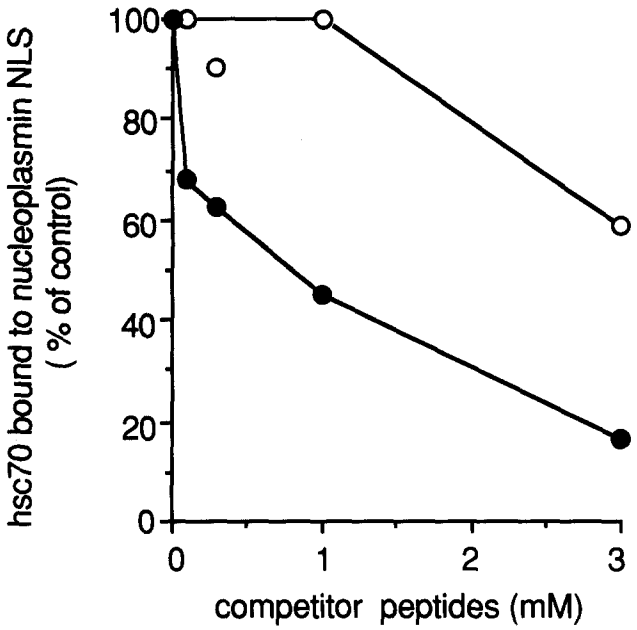

Figure 6. NLS-binding activities of bacterially expressed recombinant hsc70. Recombinant human hsc70 expressed in *E. coli* was purified by chromatography as described in Materials and Methods. For each assay, 0.5 μg of purified hsc70 labeled with ^{125}I (3 × 10^6 cpm/μg) was incubated with 15 μl of nucleoplasmin-Sepharose (correspond to 15 μg nucleoplasmin) in the presence of various amounts (0–3 mM) of $T_{(24)}$-peptide (●) or mutant $T_{(24)}$-peptide (○). After incubation, hsc70 was eluted from Sepharose with 2 mM nucleoplasmin-peptide. Eluted hsc70 was analyzed by SDS-PAGE followed by autoradiography and the amount of hsc70 was measured by densitometric scanning. The amount of hsc70 precipitated in the absence of competitive peptides was taken as 100%.

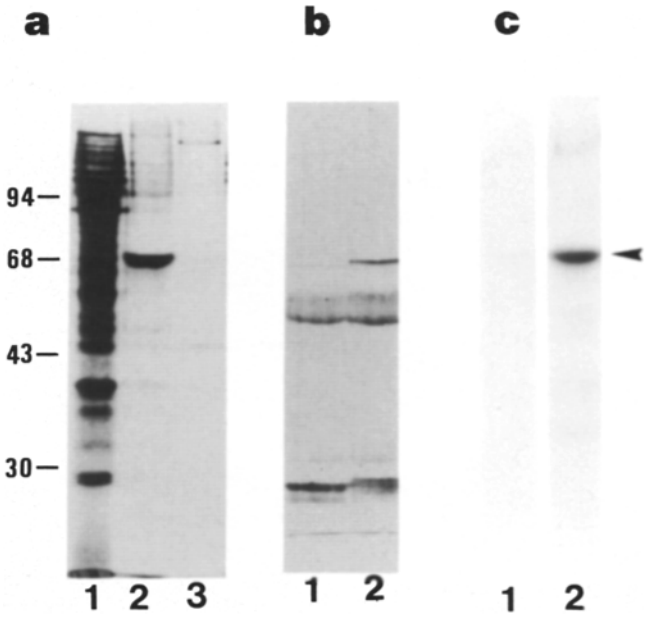

Figure 7. Recognition of hsc70 by anti-DDDED mAb Q1. (*a*) Immunoprecipitations with mAb Q1 (lane *2*) and control mAb C8 (IgM) (lane *3*) of high-salt detergent extract of nuclear envelopes isolated from [^{35}S]methionine labeled HeLa cells. Lane *1* shows the total labeled proteins in the extract. (*b*) Western blots of proteins immunoprecipitated with Q1 (lane *2*) and C8 (lane *1*) with anti-hsc70 rabbit polyclonal antibodies. (*c*) Immunoprecipitations of ^{125}I-labeled purified hsc70 with Q1 (lane *2*) and C8 (lane *1*). Molecular weight markers were as for Fig. 1. Arrowhead indicates the position of hsc70.

FITC-dextran (17.4-kD non-karyophilic molecule). Rhodamine (RITC)-labeled histone H1, RITC-lysozyme, or FITC-dextran was injected into the cytoplasm of HEL cells with affinity-purified anti-hsc70 or control IgG. After incubation for 30 min at 37°C, the cells were fixed and the localizations of the labeled molecules were determined by fluorescence microscopy. When co-injected with anti-hsc70, non-karyophilic molecules such as lysozyme and dextran rapidly migrated into the nucleus within 30 min, whereas karyophilic histone H1 was retained in the cytoplasm (Fig. 10). Co-injection of control IgG did not affect the nuclear migrations of these molecules. (Accumulation of nonkaryophilic small molecules in the nucleus, as observed here, is generally observed phenomena) (Breeuwer and Goldfarb, 1990; Yoneda et al., 1987.) These results show that the inhibitory effect of anti-hsc70 antibodies is specific to the mediated nuclear import of karyophilic proteins and that the inhibition of nuclear accumulation of karyophilic proteins is not caused by physical obstruction of nuclear pores, such as by antibody-hsc70 complexes.

70-kD heat shock proteins are thought to participate in various cellular processes (for reviews see Pelham, 1986; Rothman, 1989; and Gething and Sambrook, 1992). Injection of anti-hsc70 may have a general toxic effect on cells, such as by ATP depletion, or by killing the cells, which may affect the mediated import indirectly. To exclude these possibilities, we performed two experiments to confirm that antibody injection did not kill the cells or deplete them of energy. When the antibodies were injected with karyophilic proteins and the cells were incubated for 3 h before fixation, these proteins were found in the nuclei. Furthermore, when karyophilic proteins were injected 3 h after the antibodies, they accumulated in the nucleus within 30 min (data not shown). These results show that the inhibitory effect of antibodies was reversible and that injected cells were viable, at least with the amounts of the antibodies that could inhibit the nuclear protein import.

Next, we examined the effect of injection of anti-hsc70 on RNA synthesis in the cells. As shown in Fig. 11, the level of [^3H]uridine uptake after the injection was almost the same as that by untreated cells (and also of that of cells after injection of normal rabbit IgG). This shows that ATP was not depleted in cells after injection of a concentration of antibodies that inhibited nuclear import. Taken together, these results strongly suggest that a member of 70-kD heat-shock protein plays an important role in mediated nuclear protein import in living cells.

Discussion

We have purified a nuclear envelope-associated 69-kD protein (p69) that was originally identified by anti-DDDED antibodies as an NLS-binding protein (Imamoto-Sonobe et al., 1990). The mass, pI, cellular localization, and amino acid

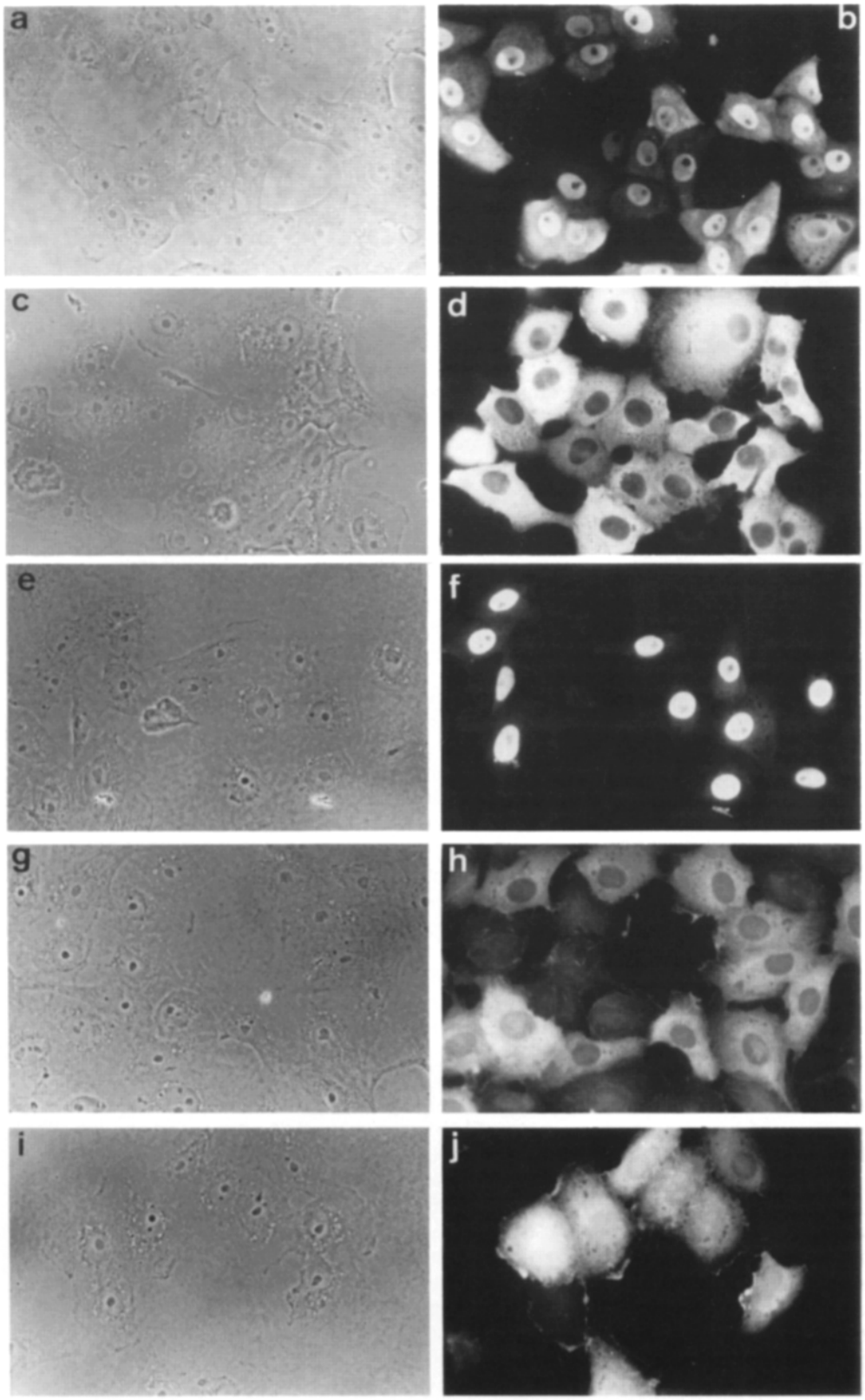

Figure 8. Effects of anti-hsc70 antibodies on nuclear transport of nucleoplasmin and SV-40 T NLS-BSA. Affinity-purified rabbit anti-hsc70 antibodies (20 mg/ml in *c*, *d*, *g*, and *h*, and 15 mg/ml in *i* and *j*) or control antibodies (20 mg/ml in *a*, *b*, *e*, and *f*) were injected with T-BSA (1.5 mg/ml in *a*, *b*, *c*, and *d*) or nucleoplasmin (0.7 mg/ml in *e*, *f*, *g*, *h*, *i*, and *j*) into the cytoplasm of HEL cells. The cells were then incubated at 37°C for 30 min before fixation. The subcellular locations of injected nuclear proteins were detected by indirect immunofluorescence staining with mouse mAbs to nucleoplasmin or sheep antibodies to BSA, and then with FITC-conjugated antibodies to mouse or sheep IgG. Procedures were as described by Yoneda et al. (1988).

sequences of the purified protein showed that it is hsc70. Hsc70 was recognized by antibody raised against DDDED-peptide (Fig. 7). Because the primary sequence of hsc70 does not contain the sequence DDDED, this antibody may recognize nonlinear epitopes of the folded hsc70 molecule. Nuclear envelope–associated hsc70, and the purified bacterially expressed recombinant hsc70 indeed showed specific NLS-binding activities (Fig. 5, *c* and *d* and Fig. 6). In the previous study, we found that p69 is membrane associated, but is not an integral membrane protein (Imamoto-Sonobe

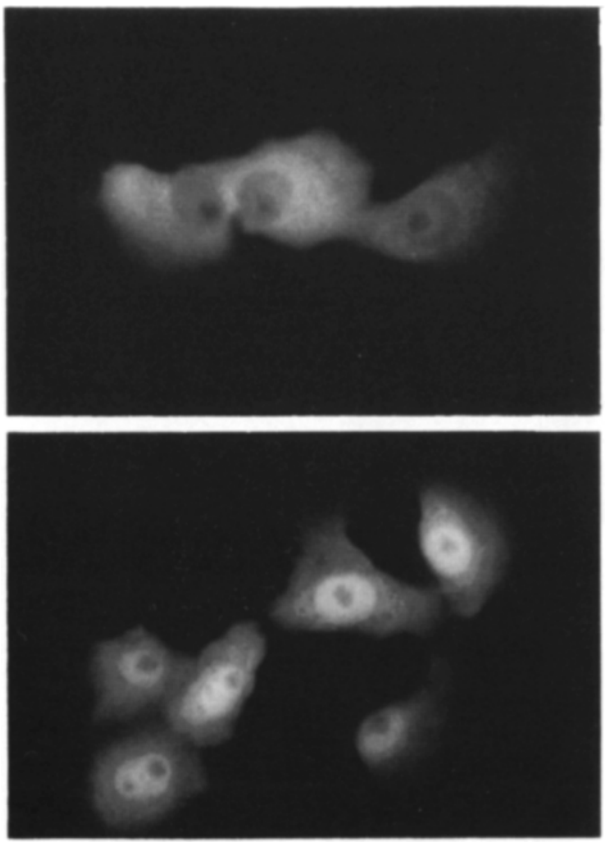

Figure 9. Absorption of the inhibitory activity of anti-hsc70 antibodies with recombinant hsc70 expressed in bacteria. Volumes of 10 μl of anti-hsc70 antibodies (18 mg/ml) incubated with 120 μg of BSA (*top*) or chromatographically purified recombinant hsc70 (*bottom*) for 4 h at 4°C were co-injected with FITC-labeled T-BSA. The cells were then incubated at 37°C for 30 min before fixation, and the subcellular locations of injected FITC-T-BSA were examined by fluorescence microscopy.

et al., 1990). p69 was recovered in the Triton X-114 nonpartitioned fraction of nuclear envelope extracts containing nonintegral membrane proteins (Fig. 1 *c*). Hsc70 is also certainly not an integral membrane protein. Moreover, a small portion of hsc70 is tightly associated with nuclear envelopes, and is extracted most effectively with buffer containing both high salt and detergent (unpublished observations). From these findings, we conclude that p69 identified previously is hsc70.

Our previous observations of predominant nuclear rim staining with anti-DDDED antibodies (together with predominant immunoabsorption activity in nuclear envelope fractions) suggest that the proteins recognized by these antibodies might localize exclusively in the nuclear envelope (Yoneda et al., 1988). Anti-DDDED antibodies detected p69 chromatographically only from the nuclear envelope fractions. However, these antibodies also recognized a considerable amount of the 69-kD protein in the nucleoplasm and DNase I supernatant on immunoblotting, suggesting that p69 may be present not only in the nuclear envelope but also diffusely throughout the nucleus (Imamoto-Sonobe et al., 1990). The present results indicate that p69 is hsc70, a protein that is localized diffusely throughout the cytoplasm and the nucleus. Then, why did the original antibodies show predominant nuclear-rim staining instead of diffusive staining throughout the cytoplasm and the nucleus? Two possible explanations can be considered: (*a*) The original anti-DDDED antibodies may have recognized a specific site (or conformation) of the hsc70 molecule that is "masked" or "unexposed" in the cytoplasm or DNase I supernatant, and thus they may have predominantly recognized a subpopulation of hsc70 that is associated with nuclear envelopes; and (*b*) the original anti-DDDED antibodies may have recognized several proteins from nuclear envelopes, one of which was p69. If this was the case, nuclear-rim staining would be stronger than diffuse cytoplasmic and nuclear stainings.

The heterogeneity or specificities of hsp70s with their targets are not well understood. Members of 70-kD heat-shock proteins are commonly thought to interact with hydrophobic domains exposed on unfolded or damaged proteins (Pelham, 1986; Beckmann et al., 1990; Flynn et al., 1991). However, the targets of cytosolic hsp70s are not limited to nascent polypeptides or damaged proteins. Hsc70 is reported to interact with a distinct sequence of clathrin light chain LCa (residues 47–71) (DeLuca-Flaherty et al., 1990). Moreover, prp73 (hsc70) is reported to bind to specific peptide sequences (KFERQ and related sequences) that target intracellular proteins for lysosomal degradation (Chiang et al., 1989). Our current results indicated that hsc70 interacts with highly positively charged NLSs, and its interaction is apparently more efficient with transport competent NLS than point mutated transport incompetent NLS.

Anti-hsc70 antibodies strongly inhibited the nuclear accumulation of various karyophilic proteins such as nucleoplasmin, SV-40 T-antigen NLS bearing BSA, and histone H1. The inhibitory activity of the antibodies was absorbed by chromatographically purified recombinant hsc70 expressed in *E. coli*. This confirms that transport inhibition was caused by anti-hsc70 antibodies, not by contaminating antibodies against polypeptides not related to hsc70. Most importantly, the inhibition was specific to signal mediated import. These antibodies did not inhibit the diffusive import of nonkaryophilic small molecules, such as small basic protein lysozyme. These results also showed that nuclear pores were not physically obstructed by, for example, antibody-hsc70 complexes. The injection of anti-hsc70, at protein concentrations that completely inhibited nuclear import, did not kill the injected cells because these cells were viable and the effects were reversible. Moreover, the injection of antibodies did not cause depletion of cellular ATP, because RNA synthesis continued normally in the cells after injection of these antibodies. Thus although hsc70 is considered to participate in various cellular functions, we conclude from our results that the inhibition of nuclear transport by anti-hsc70 antibodies is not the result of pleiotropic physiological effects.

A number of NLS-binding proteins have been identified (Adam et al., 1989; Yamasaki et al., 1989; Li and Thomas, 1989; Meier and Blobel, 1990; Lee and Melese, 1989; Silver et al., 1989). The relationships of all these NLS-binding proteins are not yet known, but none of these reported proteins are members of the 70-kD heat-shock protein family. In spite of the use of the same NLS (for example SV-40 T-antigen NLS) as probe, different strategies (cross-linking, ligand blotting, and in our case anti-peptide antibodies) and different experimental conditions have led to the identifica-

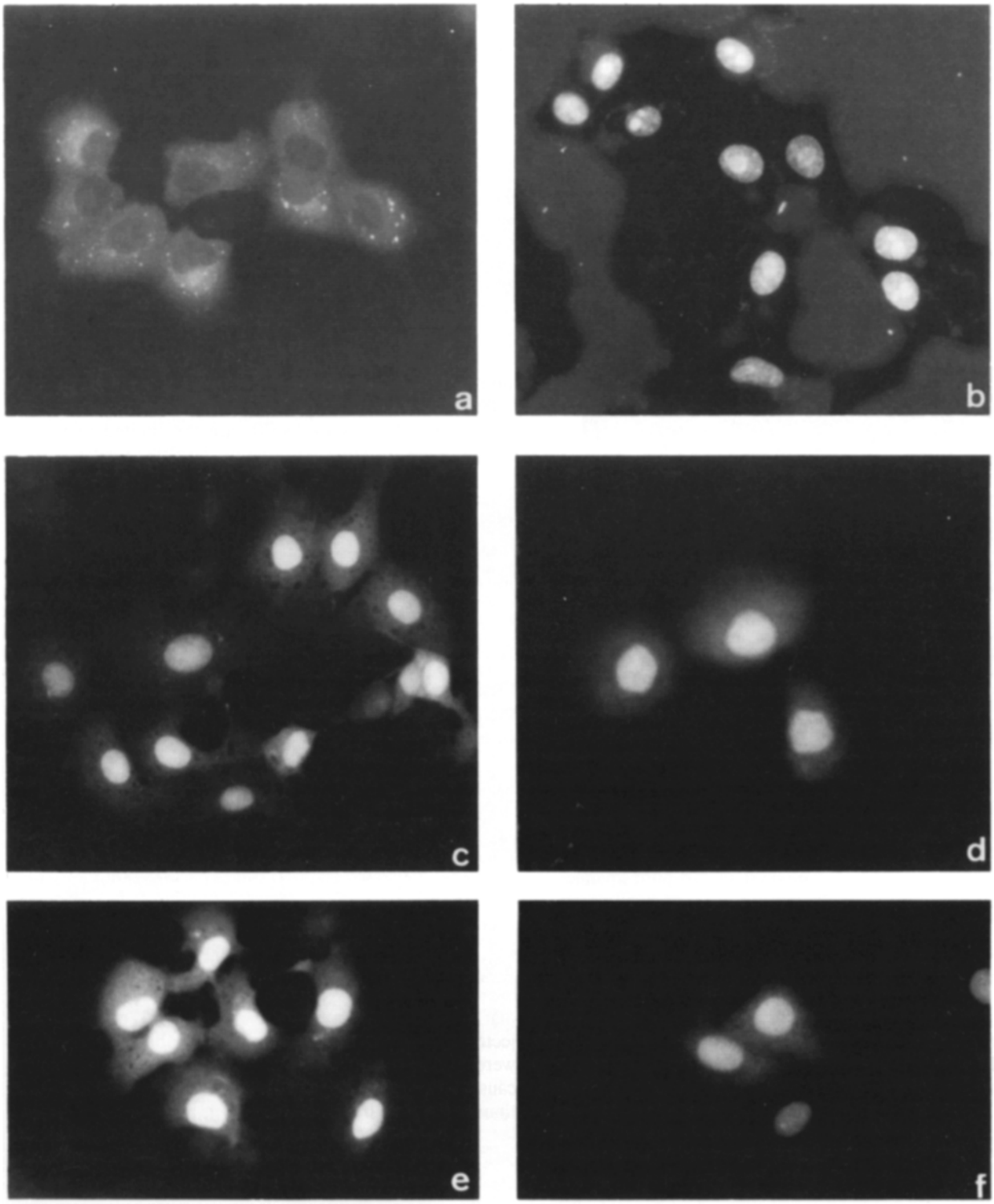

Figure 10. Effects of anti-hsc70 on nuclear import of small karyophilic and non-karyophilic molecules. Affinity-purified rabbit anti-hsc70 antibodies (20 mg/ml in *a*, *c*, and *d*) or control antibodies (20 mg/ml in *b*, *e*, and *f*) were injected with RITC-labeled histone H1 (*a* and *b*). RITC-lysozyme (*c* and *e*) or FITC-dextran (*d* and *f*). The cells were then incubated at 37°C for 30 min before fixation and the subcellular locations of injected molecules were examined by immunofluorescence microscopy.

tions of different NLS-binding proteins. Therefore, functional analyses are necessary to define the actual involvements of these proteins in nuclear transport. Adam and Gerace (1991) purified bovine 54–56-kD cytoplasmic NLS-binding proteins that support nuclear import in semi-permeabilized cells. Stochaj and Silver (1992) showed that antibodies against 70-kD cytoplasmic NLS-binding protein inhibit nuclear transport in semi-permeabilized cells. We

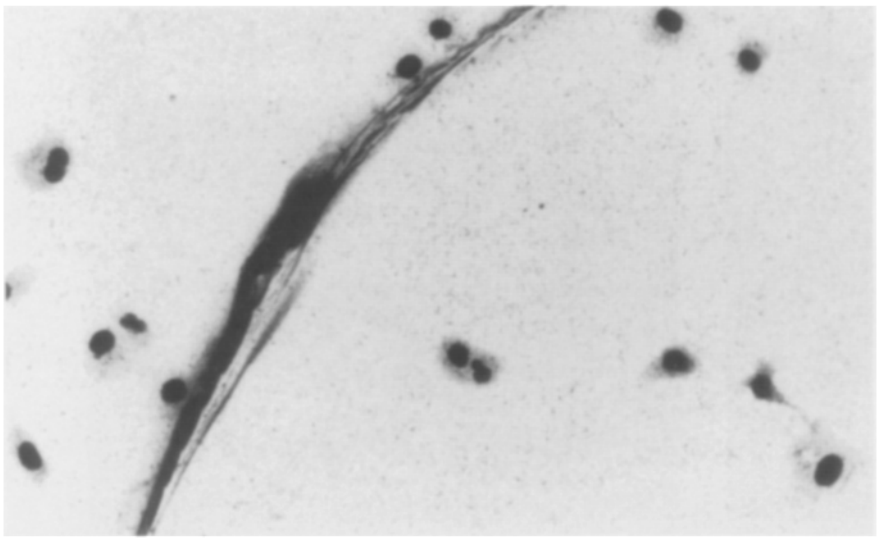

Figure 11. Effects of anti-hsc70 on RNA synthesis. HEL cells were plated on coverslips with small circles cut with a diamond knife. Immediately after injection of anti-hsc70 antibodies (at a protein concentration that completely inhibited nuclear protein import) into the cytoplasm of the cells in the small circles, [^{3}H]uridine was added to the culture medium at a final concentration of 30 μCi/ml. After labeling for 30 min at 37°C, cells were fixed, and autoradiographed as described previously (Yoneda et al., 1987). The cells on the right side of the groove received an injection of anti-hsc70 antibodies whereas those on the left did not.

have identified hsc70 as an NLS-binding protein. Antibodies against hsc70 inhibited nuclear transport in living cells.

The presence of a number of NLS-binding proteins raises the possibility of the existence of multiple NLS receptors for SV-40 T antigen-like NLS, or since nuclear transport is a multi-step process, the possibility that karyophilic proteins may need to interact with two or more NLS-binding proteins before they are transported into the nucleus. As the present results show that hsc70 has affinity to NLSs, it is interesting to consider the following possibilities for how this protein participates in nuclear transport through interaction with NLSs. First, hsc70 could be one of the multiple NLS-receptors that carries karyophilic proteins from the cytoplasm into the nucleus. Second, hsc70 could promote correct assembly (or disassembly) between NLS and NLS receptors by interacting with NLSs. Third, hsc70 could be the ATPase involved in NLS-dependent dilation of the pore channel. Further studies on factors that co-operate with hsc70 in nuclear transport are required to determine its actual role.

We thank Dr. M. Nakanishi for discussions and reading through this manuscript. We also thank Dr. David S. Goldfarb for discussions and suggestions.

This work was supported by grants from the Ministry of Education, Science and Culture (grant 04259101), and the Science and Technology Agency of Japan.

Received for publication 6 January 1992 and in revised form 4 August 1992.

Note Added in Proof. During submission of this paper, a study by Shi, Y., and J. O. Thomas (1992. Mol. Cell. Biol. 12:2186) was published, which demonstrated that hsp70 and hsc70 support the nuclear import of nucleoplasmin in semi-permeabilized cells.

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