Journal of Cellular Biochemistry 74:145–151 (1999)

FAST TRACK

Reassembling Proteins and Chaperones in Human Nuclear Matrix Protein Fractions

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Abstract To detect putative filament forming components, nuclear matrix proteins were searched for proteins extensively reassembling from urea solution. Eight proteins, ubiquitously occurring in various human cell types, but not apparent in the cytosol, were registered by means of two-dimensional gel electrophoresis. They consisted of a protein exhibiting a novel amino acid sequence; of nuclear lamin B2, RbAp46, and RbAp48; and of four as yet unknown proteins. Furthermore, partial sequencing, mass spectrometry, and immunodetection of proteins demonstrated the presence of molecular chaperones and protein folding catalysts in the nuclear matrix fractions. In addition to a TCP-1-related protein, certain members of the heat shock, PDI, and calreticulin family of proteins were detected. On the basis of the absence of several other heat shock proteins in the nuclear matrix fraction, a general contamination by cytoplasmic chaperones appears unlikely. J. Cell. Biochem. 74:145–151, 1999.

Key words: nuclear matrix proteins; reassembling nuclear proteins; chaperones; retinoblastoma binding proteins; two-dimensional polyacrylamide gel electrophoresis

An unresolved topic is whether the spatial organization of the cell nucleus is mediated by protein structures analogous to the filaments observed in the cytoplasm, or indirectly accomplished by dynamic protein-chromatin interactions [Marshall et al., 1997]. Nuclear filaments were observed by electron microscopic examination of nuclear matrix preparations [Fey et al., 1986], but no filamentous structures of the nucleus could be visualized by immunofluorescence or immunoelectronmicroscopy of intact nuclei. Proteome analysis of nuclear matrix protein fractions showed the prominent presence of components of the RNA transcription, processing, and transport machinery [Mattern et al., 1996; Gerner et al., 1998], which have not been reported to assemble filamentous structures. Only recently, the mitotic apparatus protein (NuMA) could be demonstrated to establish a regular nuclear lattice when being overexpressed [Gueth-Hallonet et al., 1998]. However, many nuclear matrix constituents remain unknown [for reviews, see Berezney and Jeon, 1995].

Protein constituents of cytofilaments do have the ability to establish protein filaments, allowing the specific reassembly of such filamentous structures by dialysis of urea-solubilized protein fractions against imidazole-containing reassembly buffer [Zackroff et al., 1982]. In search of potential filament-forming nuclear matrix proteins, we investigated nuclear matrix proteins with a capability to reassemble during dialysis similar to cytofilament proteins. For this purpose, we solubilized nuclear matrix-intermediate filament protein fractions in urea buffer and analyzed the soluble and insoluble protein fractions obtained after dialysis by comparative two-dimensional gel electrophoresis.

By this means, several nuclear matrix proteins could be discriminated as quantitatively precipitated during dialysis, a property otherwise observed only with intermediate filament proteins and actin. Interestingly, none of these nuclear matrix proteins was apparent in cytosol protein fractions. Further analysis of the respective proteins permitted identification of the retinoblastoma binding proteins RbAp46&48

Grant sponsor: Herzfelder Stiftung, Vienna, Austria.

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Received 26 March 1999; Accepted 29 March 1999

and Lamin B2. In addition, a partial amino acid sequence of a novel protein not represented in current protein databases could be obtained. Mass spectrometry data indicate further novel proteins amongst this group.

By contrast, most nuclear matrix constituents did not precipitate quantitatively during dialysis. Several of these, which were also ubiquitously present in the nuclear matrix protein fractions of various cell types, were also found to occur in the respective cytosol protein fractions and could be identified as chaperones. In conclusion, we newly classified the nuclear matrix protein composition by consideration of the reassembling capability of proteins and their subcellular distribution. In addition to a group of several chaperones present in the nuclear matrix protein fraction, we present a group of proteins potentially involved in higher structure organization of the nucleus.

MATERIALS AND METHODS

Cells

For the isolation of human peripheral blood mononuclear cells heparinized blood was mixed with 2 vol Hank's balanced salt solution (HBSS), layered on Ficoll Paque (Pharmacia, Uppsala, Sweden), and centrifuged. The interface cells, comprising B and T lymphocytes and monocytes, were washed in HBSS.

Preparation of Subcellular Fractions

Human peripheral blood cells were lysed by suspension in 0.05% Nonidet P-40 (NP-40) (Sigma, St. Louis, MO) in hypotonic buffer. Crude nuclei were obtained by centrifugation at 400*g*, the supernatant was again centrifuged at 100,000*g* for 1 h to obtain the cytosol freed from insoluble cellular remnants. Subsequently, the cytosol proteins were ethanol precipitated.

Nuclear matrices of leukocytes were prepared as detailed before [Gerner and Sauermann, 1999]. In short, crude nuclei were pelleted through a 300 mM sucrose cushion, sheared in 1.5 M sucrose in a tight-fitting potter and centrifuged through a 2 M sucrose cushion. The nuclei were resuspended in 2 mM vanadyl ribonucleoside complex (VR)(Gibco BRL, Gaithersburg, MD) in 1 M sucrose, and then exposed to 0.2% sodium deoxycholate (DOC) (Sigma) and 0.4% Tween 40 (Sigma). DNA was digested with 100 U/ml deoxyribonuclease I (DNase I, from bovine pancreas, EC 3.1.21.1, Sigma) at 22C for 30 min. After centrifugation, the residual nuclear pellet was extracted with 250 mM ammonium sulfate and washed, yielding the nuclear matrix (protein) fraction. Nuclear matrices of cultured and of tissue cells were prepared by modified procedures [Gerner et al., 1998].

Detection of Reassembling Nuclear Matrix Proteins

The nuclear matrix-intermediate filament fraction was prepared according to Fey and Penman [1988]. In short, blood cells were lysed in 0.5% Triton X-100, 2 mM vanadyl ribonucleoside (VR), and the nuclei exposed to 0.5% (DOC), 1% Tween-40. After chromatin digestion and ammonium sulfate extraction, the proteins were dissolved in 8 M urea, 20 mM 4-morpholineethanesulfonic acid (pH 6.6), 1 mM EGTA, 0.1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1% 2-mercaptoethanol (disassembly buffer), and separated from insoluble material by centrifugation at 250,000*g* for 1 h at 4°C [Zackroff et al., 1982]. The supernatant was dialyzed for 12 h at 25°C against 1,000 vol of 0.15 M KCl, 25 mM imidazol.HCl (pH 7.1), 5 mM $MgSO_4$, 2 mM dithiothreitol (DTT), 125 µM EGTA, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (reassembly buffer). Finally, centrifugation at 150,000*g* for 90 min at 25°C yielded the pellet containing the reassembled proteins. The supernatant was ethanol precipitated.

Two-Dimensional Polyacrylamide Gel Electrophoresis and Image Analysis

High-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), blotting, silver staining of proteins, and computer-assisted image analysis were carried out as reported before [Gerner et al., 1998]. The protein samples were dissolved in 10 M urea, 4% CHAPS, 0.5% sodium doddecyl sulfate (SDS), and 100 mM DTT supplemented with 2% ampholytes) separated in the Protean II xi electrophoresis system (BioRad, Richmond, CA), using the method of Hochstrasser et al. [1988].

Identification of Proteins

Amino acid sequencing and matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry of trypsin hydroly-

sates was performed as reported previously for hnRNPs H and H′ [Holzmann et al., 1997]. For Western blotting, proteins were electrophoretically transferred onto nitrocellulose membranes (0.2 µm). The monoclonal antibody specific for hsp60 was purchased from StressGen Biotechnologies (Victoria, Canada). The monoclonal antibody specific for hsp70 and the polyclonal antibody specific for hsp90 was purchased from NeoMarkers (Union City, CA). Protein identification was confirmed by comparison of the obtained molecular weight/pI data with those published in the Human 2-D PAGE Databases for proteome analysis in health and disease by the Danish Centre For Human Genome Research at http://biosun.biobase.dk [see also Celis et al., 1995].

RESULTS

Characterization of Nuclear Matrix Proteins Capable of Reassembling

These experiments were performed with human peripheral blood mononuclear cells exhibiting low amounts of cytofilaments. Following the procedures of Fey and Penman [1988] and Zackroff et al. [1982] in the reassembly experiments, nuclear matrix-intermediate filament samples were dissolved in urea buffer. Reassembly of proteins was induced by dialysis against isotonic imidazole buffer and the resulting insoluble material collected by centrifugation. Figure 1 shows the protein patterns of the pellet and of the supernatant fraction after high- resolution 2-D gel electrophoresis ranging between

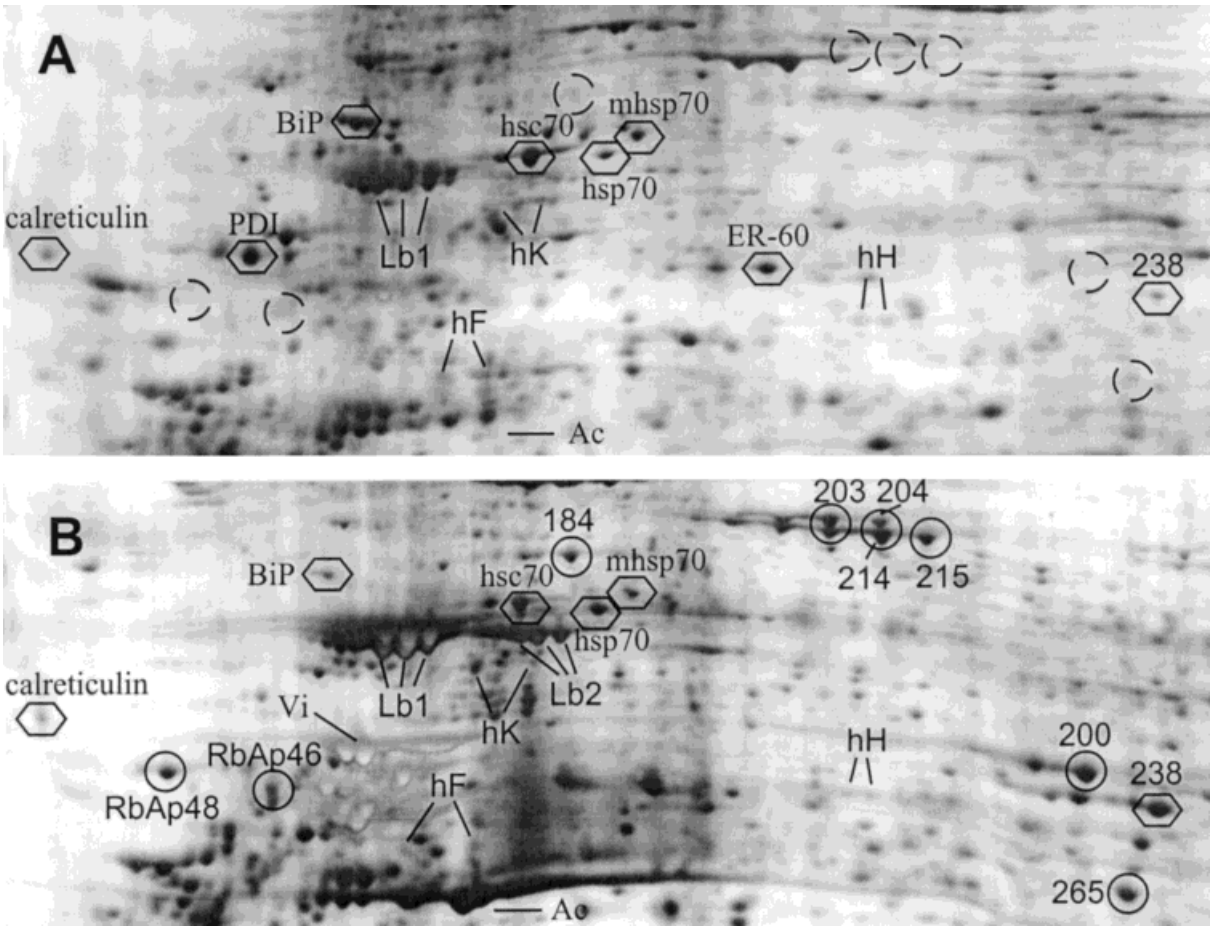

Fig. 1. Reassembly of nuclear matrix proteins. Silver-stained two-dimensional (2D) gel sections of protein fractions obtained after dialysis of nuclear matrix-intermediate filament protein fraction obtained from human peripheral blood mononuclear cells. **A:** Supernatant matrix-intermediate. **B:** Pelleted (reassembling) proteins. Circles, reassembling common nuclear matrix proteins (as defined in text); open circles, location of missing reassembling proteins; hexagons, chaperones as indicated. Ac, actin; hF, hH, hK, heterogeneous nuclear ribonucleoproteins F, H, K; Lb1, Lb2, nuclear lamins B1, B2; Vi, vimentin; 184, 200, 203, 204, 214, 215, 259, human nuclear matrix proteins 184, 200, 203, 204, 214, 215, 238.

TABLE I. Reassembling Proteins in the Human Nuclear Matrix Protein Fraction[a]

Proteins	Characterization (+)	Cell fraction		Dialysis fraction	
		Cytosol	Nuclear matrix	Soluble	Pellet
hNMP 184	83 kDa/pI 5.4; (M&P: u)	−	+	−	+
hNMP 200	57 kDa/pI 6.37; A: KYIAENGTD, (M&P: u)	−	+	−	+
hNMP 203,204	93 kDa/pI 5.8; 93/5.9; (M&P: u)	−	+	−	+
hNMP 214,215	90/93 kDa/pI 5.9/6.0; (M&P: u)	−	+	−	+
hNMP 265	47 kDa/pI 6.42; (M&P: u)	−	+	−	+
RbAp 46	49 kDa/pI 4.8	−	+	−	+
RbAp 48	51 kDa/pI 4.45; A: GEFGGFGSVT/SG	−	+	−	+
Lamin B2	65 kDa/pI 5.35; A, I [Gerner et al., 1998]	−	+	−	+

[a](+) Protein characterized by A, partial amino acid sequencing; I, immunodetection; P, position in 2-D gels in relation to published 2-D-PAGE databases [Celis et al., 1995]; M&P, mass spectrometry data combined with observed position in 2-D gels (kDa/pI). u, unrelated to known (published) sequences; hNMP, human nuclear matrix protein.

TABLE II. Chaperones in the Nuclear Matrix Protein Fraction[a]

Family	Protein	Characterization	Cell fraction		Dialysis fraction	
			Cytosol	Nuclear matrix	Soluble	Pellet
TCP-1 related	hNMP 238 (TIP49)	54 kDa/pI 6.5; A, I [Holzmann et al., 1998]	+	+	+	+
Heat shock	hsc 70	67 kDa/pI 5.3; A: SQIHDIV-LVGGSTR, TTSYVAF; I	++	++	++	++
	hsp 70	66 kDa/pI 5.5; I; M: 1018.3, 1109.2, 1134.6, 1197.5, 1228.1, 1329.5, 1487.9, 1658.5, 1676.9, 1687.9, 2787.0, 3003.8	+	+	+	+
	mhsp 70 (Grp 75)	68 kDa/pI 5.52; I; M: 864.8, 1002.5, 1018.6, 1194.8, 1211.9, 1291.4, 1363.5, 1593.1, 1845.3	+	+	+	+
	BiP (Grp 78)	68 kDa/pI 4.9, (P)	++	++	++	+
PDI	PDI	57 kDa/pI 4.6, (P)	++	+	++	−
	ER-60 (Grp 58)	58 kDa/pI 6.0; A: SDVLELTDD	++	++	++	−
Calreticulin	Calreticulin	57 kDa/pI 4.2; A: EQFLDG-DGWTS	++	+	+	+

[a]Designations as in Table I. Alternative names of proteins in brackets.

40–100 kDa and pI 4.0–6.5. The specific patterns of the silver-stained protein spots annotated in Figure 1 and listed in Tables I and II were recognized in at least 10 independent experiments. In addition, these spots were identified in nuclear matrices isolated from various sources, such as human tissues (kidney, liver, lung, spleen), cultured cells (various leukemia and carcinoma cell lines), and subtypes of hematopoietic cells (lymphocytes, monocytes, neutrophils) (not shown; see Gerner et al., 1998; Gerner and Sauermann, 1999). Therefore, we regard these proteins as common nuclear matrix proteins. Protein identification was accomplished by partial amino acid sequencing, mass spectrometry, immunoblotting, or their characteristic gel position, with respect to the human keratinocyte two-dimensional protein databases [Celis et al., 1995] (Tables I and II).

After dialysis, several proteins were found in both precipitated and soluble protein fractions, such as heterogeneous nuclear ribonucleoproteins F, H, and K (Fig. 1). However, as exemplified with cells containing high amounts of cytokeratins (not shown), proteins with reassembling capability such as cytokeratins were found almost quantitatively in the pelleted protein fraction, but not in the supernatant (or only in trace amounts). Several other proteins of the nuclear matrix-intermediate filament protein fractions

were found in a similar exclusive distribution. These proteins are in the following designated as reassembling proteins.

Characteristics and Identification of Reassembling Nuclear Matrix Proteins

The reassembling nuclear matrix proteins shown in Table I were excluded to represent cytokeratins, as they were not stained with a pan anti-cytokeratin antibody (not shown). In addition, their molecular weight and pI values, as well as mass spectrometry data obtained from each, did not match with those corresponding to known cytokeratin subtypes or corresponding degradation products.

Comparison of cytosol and nuclear matrix 2D spot patterns and comigration experiments with both protein fractions (not shown) showed that the reassembling nuclear matrix proteins were not detectable in the cytosol protein fraction (Fig. 2A).

By partial amino acid sequencing, one protein spot was identified as retinoblastoma binding protein 48 (RbAp48) (Table I, Fig. 2B). Furthermore, a published 2-D spot pattern of RbAp48 and RbAp46 permitted identification of RbAp46 [Qian et al., 1993]. Nuclear lamin B2 also appeared as reassembling protein. This finding is in contrast to lamin A, B1 and C, which were found in both dialysis pellet and supernatant protein fractions (Fig. 1; data not shown).

Partial amino acid sequence showed the sequence KYIAENGTD for hNMP-200. Databank searches failed to detect homology to known proteins. Meanwhile, however, its cDNA was cloned and sequenced, as will be presented elsewhere (manuscript in preparation). Similarly, protein hNMP 259 was recognized as novel nuclear matrix constituent (manuscript in preparation). Furthermore, mass spectrometry analysis of the hydrolyzed hNMPs 184, 203,

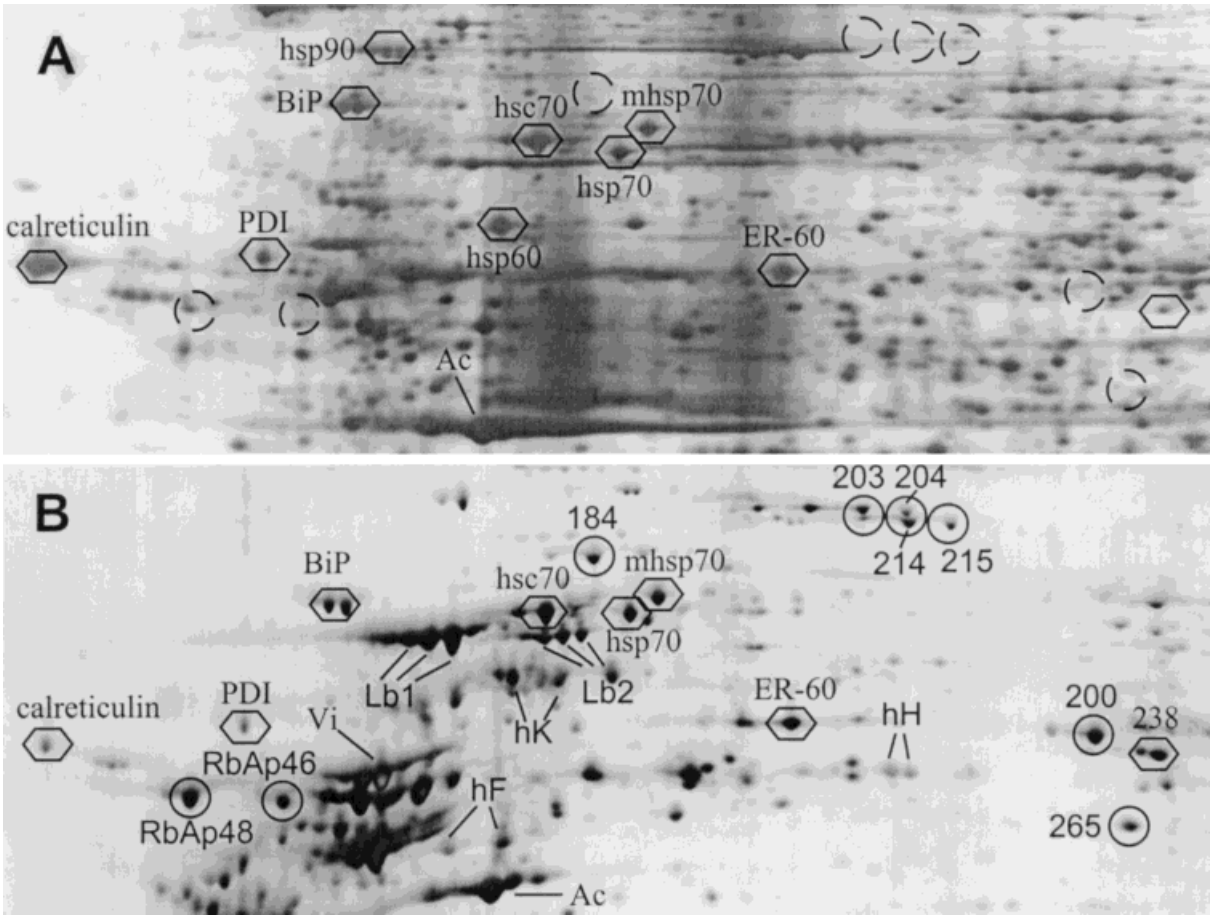

Fig. 2. Proteins of the nuclear matrix and cytosol fraction. **A:** Cytosol. **B:** Nuclear matrix. Designations as in Fig.1.

204, 214, and 215 indicated that they represent currently unknown proteins (not shown).

Chaperones in the Nuclear Matrix Protein Fraction

In contrast to the reassembling nuclear matrix proteins, several proteins of the nuclear matrix fraction were found to occur in the cytosol fraction as well (Fig. 2), as confirmed by comigration experiments (not shown). These proteins could be identified as molecular chaperones, including four members of the heat shock family of proteins, in addition to two members of the protein disulfide isomerase (PDI) family of proteins, and calreticulin (Table II). These ubiquitously occurring spots are designated by hexagons in Figures 1 and 2, and the mode of their identification is given in Table II.

To avoid aggregation of nuclei, low detergent concentrations were being applied during the nuclear matrix preparation protocol (see Materials and Methods), suggesting inefficient extraction as a potential cause for the presence of chaperones. However, application of high detergent concentrations as applied during the preparation the nuclear matrix-intermediate filament protein fraction did not permit extraction of chaperones with higher efficiency, as can be observed in the dialysis supernatant and pellet (Figs. 1, 2). Therefore, the number of chaperones present in the nuclear matrix fraction was not critically dependent on the detergent concentrations employed to extract nuclei.

DISCUSSION

To remove cytofilament proteins selectively, a well-established nuclear matrix preparation protocol uses a dialysis step of a solubilized nuclear matrix-intermediate filament protein fraction against a reassembly buffer, which was designed to allow cytofilament proteins preferentially to reassemble and precipitate [Fey and Penman, 1988]. This separation principle relies on the assumption, that cytofilament proteins show an inherent tendency to repolymerize, whereas nuclear matrix proteins do not. In this article, we demonstrated that, indeed, several nuclear proteins, classified by their absence in the cytosol protein fraction, were found quantitatively precipitated by this dialysis step. In addition, these proteins were excluded to represent known cytofilament proteins. Therefore, this procedure was used to classify a group of nuclear proteins owing a potential capability to reassemble. Alternatively, the observed quantitative reassembly of proteins during the dialysis step might be explained by establishment of macromolecular complexes, by high affinity to polymerized proteins or simply by high insolubility of the respective proteins. However, any of these properties would justify the assumption of a potential capability of these proteins to establish or take part in higher order structures of the nucleus.

In contrast to this group of proteins, another group of proteins that did not reassemble was characterized by their ubiquitous presence in nuclear matrix and cytosol protein fractions. Subsequent protein analysis by various methods allowed identification of several chaperones, which have been described to localize in the cytoplasm [for reviews, see Gething, 1997]. However, calreticulin and members of the hsp70 family have been reported to be localized in almost all subcellular compartments including the nuclear matrix [Pouchelet et al., 1983; Jethmalani et al., 1997]. In addition, hsp70 was suggested to contribute essentially to protein translocation into nuclei [Shulga et al., 1996]. The nuclear matrix protein hNMP 238 was recently described as being related to subunits of T-complex protein 1 (TCP-1) [Holzmann et al., 1998]. Interestingly, another member of this complex, the TCP-1 γ subunit, has been identified as a constituent of the nuclear matrix [Joly et al., 1994]. However, it remains to be established whether the chaperone molecules found in the nuclear matrix protein fraction might play a role in assembly, constitution or function of the nuclear framework. Alternatively, several of the chaperones may be proteins copurifying during the isolation procedure. This may apply for BiP, for example, which has been described to bind to hydrophobic patches of incorrectly folded proteins which may be obtained after treatment of the nuclei with detergent [Schmitz et el., 1995]. By contrast, the chaperones hsp60, hsp90 (Fig. 2), and hsp27 (not shown) were not detectable in the nuclear matrix fraction, arguing against a generally occurring unspecific copurification of cytosolic chaperones with the nuclear matrix.

Referring to the identified reassembling nuclear matrix proteins, the retinoblastoma binding proteins RbAp46 and RbAp48 have not yet been reported before as being constituents of the nuclear matrix. RbAp48 has been described as part of the chromatin assembly fac-

tor 1 (CAF-1) and is a member of a conserved subfamily of WD-repeat proteins, which have been suggested to function in chromatin assembly, histone acetylation, deacetylation or generally as histone chaperones [Verreault et al., 1996]. The finding of lamin B2 among these proteins might not be surprising, as B-type nuclear lamins are expressed in all cells and not only appear at the nuclear periphery, but in nucleoplasmic foci as well [Moir et al., 1994]. However, lamins A, B1, and C were found to behave differently, compared with lamin B2. Functional differences between B-type lamins has been suggested to relate to differential interactions with intermediate filament proteins, which could explain our findings. Another member of the reassembling nuclear matrix proteins, hNMP-200, was found to represent a novel protein. This appears to be highly significant, as this protein was present in virtually all cell types investigated with prominent spot intensity (data not shown). Mass spectrometry data derived from hNMPs 184, 203, 204, 214, and 215 gave no significant match, in comparison with calculated data from known nuclear proteins with similar molecular weight and pI (not shown), these proteins might therefore represent unknown proteins as well. In conclusion, we further characterized the nuclear matrix protein composition and presented novel protein candidates that might be involved in higher-order structures inside the nucleus.

ACKNOWLEDGMENTS

We thank Mrs. Editha Bayer for skillful technical assistance.

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