The β-isoform of heat shock protein hsp-90 is structurally related with human microtubule-interacting protein Mip-90

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Abstract Through major research advances in the study of cytoskeletal organization, an integrated view of the complexity of this system has emerged. Recent findings on the microtubule-interacting protein Mip-90, which associates with microtubules and actin filaments in different cell domains, have shed light on its roles in cytoskeletal regulation. In order to study structural features of Mip-90, we sequenced several peptide fragments. A comparative sequence analysis revealed a high degree of similarity between the primary structure of this protein and the human heat shock protein of 90 kDa (hsp-90). Taken together, the present studies indicate the identity between Mip-90 and the the β-isoform of hsp-90 (hsp-90β). Western blot assays with an anti-hsp-90 monoclonal antibody showed cross-reactivity of hsp-90 and Mip-90 affinity purified from HeLa cells. Furthermore, the observed structural identity of Mip-90 with the hsp-90β was sustained by immunoblot assays using monoclonal antibodies that specifically recognize the α- and β-forms of hsp-90. Comparative fingerprinting analysis, along with the evidence of a remarkably similar biochemical behavior of both hsp-90 and Mip-90 in different affinity chromatographic systems, supported these observations. These studies, along with previous investigations, provide new data to elucidate the functional significance of these interesting cellular components and its relationships with other proteins linked to the cell architecture.

Key words: Microtubule interacting protein-90; Protein domain; Sequence homology; Heat shock protein-90; Common epitope; Functional feature

1. Introduction

There is increasing evidence that microtubule-associated proteins (MAPs) play a major role in regulating the interaction patterns that define cytoskeleton organization [1,2]. However, the functional versatility of the cytoskeletal structure and its involvement in morphogenetic events of cells suggests that, besides MAPs, a complex set of proteins participates in modulating cytoskeleton organization at the spatial and temporal levels [2–6]. In the search for novel microtubule-interacting proteins of functional relevance, a 90 kDa protein, designated Mip-90 was identified as a major soluble component in HeLa, N2A neuroblastoma cells and human fibroblasts [6]. Mip-90 associates reversibly with microtubules, and binds to the C-terminal β(422–434) and β(434–442) tubulin fragments, common sites for the interaction of several MAPs [2]. In addition, Mip-90 forms complexes with calmodulin in a Ca^{2+}-dependent fashion [6]. Immunofluorescence studies revealed co-localization of this protein with microtubules in various domains of interphase cells. Recent studies established that Mip-90 interacts with actin, a finding that contributes to explain the functional versatility of this protein, and its modulatory role in the organization of the actin/microtubule networks [5].

In order to gain more information regarding Mip-90's structure we have sequenced isolated fragments of this protein. The peptide sequences showed 100% homology with equivalent fragments of the β-form of human hsp-90 and a lower similarity with fragments from the α-isoform [7]. This finding was supported by: (a) Western blot assays using different antibodies revealed cross-reactivity of Mip-90, purified from HeLa cells, with the β-isoform of hsp-90; (b) comparison of peptide maps of both proteins.

Hsp-90 is an abundant, essential, cytosolic protein (under normal conditions), that increases significantly under stress conditions [8,9]. Hsp-90 has been directly involved in a wide variety of cell functions, including a chaperone activity for conformational processing of proteins, modulation of steroid receptors and association with the cytoskeleton [10–13]. The present data open new avenues to further understand functional aspects of hsp-90, especially in regard to differential roles of hsp-90 isoforms in modulating cytoskeletal interactions. On the other hand, the data on the structural and immunological similarities between Mip-90 and hsp-90 provide a background of information to re-examine structure-function relationships of these important cellular proteins.

2. Materials and methods

2.1. Cell culture

HeLa cells were used as a source of Mip-90 and hsp-90 for the biochemical and immunological studies. The human fibroblasts cells used throughout immunofluorescence studies were primary cultured originated from samples of scrotal epithelium [5]. Cells were grown in a 5% CO_2 atmosphere, in 150-mm plastic tissue culture dishes, containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum at 37°C [5,6].

2.2. Protein purification

Mip-90 was purified as described previously [6]. The heat shock protein hsp-90 was purified following the procedure of Welch and Feramisco [14].

2.3. Antibodies

An affinity-purified polyclonal antibody against Mip-90 was used throughout these studies. The specificity of the antibody to Mip-90 has been shown in previous reports [5,6]. The monoclonal antibodies against hsp-90 were obtained from Sigma Chemical Company (St. Louis, MO, USA) and from StressGene (Biotechnology, Victoria, B.C., Canada). Monoclonal antibodies that specifically recognize α- or β-isoforms of hsp-90 were kindly donated by Prof. Nemoto (Iwate Medical University, Japan). Fluorescein-conjugated secondary

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antibody, mouse IgG against rabbit IgG, was obtained from Cappel (Organon Teknika Corp, West Chester, PA, USA), and rhodamine-conjugated rabbit IgG against mouse IgG was purchased from Sigma Chemical Company (St. Louis, MO, USA). Peroxidase-conjugated antibodies were obtained from Amersham Life Science (UK).

2.4. Sequence studies on Mip-90

Mip-90 fraction purified from calmodulin affinity columns was cleaved with a sequencing grade C-terminal lysine endoproteinase (Promega, Madison, WI, USA) using a Protein Fingerprint System (Promega). The fragment products were fractionated in 15% SDS-polyacrylamide gels, electrotransferred into PDVF membranes and stained with a solution of 50% methanol/Coomassie brilliant blue. Single bands were cut and micro-sequenced using an Applied Biosystems model 470A protein sequenator. The analysis of sequence homology was carried out by using the FASTA Program [15] and local similarity Program LALIGN [16].

2.5. Peptide map analysis

Mip-90 and hsp-90 proteins were digested with lysine (Lys-C) and glutamic (Glu-C) endoproteinases, using the Protein Fingerprint System (Promega), which is based on the procedure described by Cleveland et al. [17].

2.6. Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [18]. For Western blot assays, PDVF membranes were blocked in buffer TST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.05% Tween 20) containing 3% BSA at room temperature for 2 h, washed three times in TST and incubated 2–3 h with the respective anti-Mip-90 or anti-hsp-90 antibodies diluted in TST/1% BSA. The membranes were washed three times for 10 min each in TST, and incubated at room temperature for 2 h with second antibodies conjugated with horseradish peroxidase. A chemiluminescence system (Amersham) or diaminobenzidine as the enzyme substrate were used to detect bound antibodies [19].

2.7. Indirect immunofluorescence studies

Cells grown on coverslips were permeabilized with 0.5% Triton X-100 in microtubule assembly buffer (0.1 M MES, pH 6.8, 1 mM EGTA and 1.5 mM $MgCl_2$) for 1 min at room temperature and fixed in methanol at −20°C for 10 min as described [6]. Fixed cells were incubated with PBS and incubated 1 h in blocking solution (1% BSA in PBS). Cells were incubated with affinity-purified polyclonal anti-Mip-90 antibody (1:50) or monoclonal antibodies against hsp-90α and hsp-90β as primary antibodies [20,21]. Fluorescein-conjugated mouse IgG against rabbit IgG and rhodamine-conjugated rabbit IgG against mouse IgG, previously centrifuged at 126 000×*g* in a Beckman Airfuge, were used as secondary antibodies. Cells were incubated with primary or secondary antibodies for 1 h at room temperature. Cells were rinsed in PBS, mounted in DABCO/Mowiol and examined by conventional microscopy (100× objective, Carl Zeiss Axiovert, Germany).

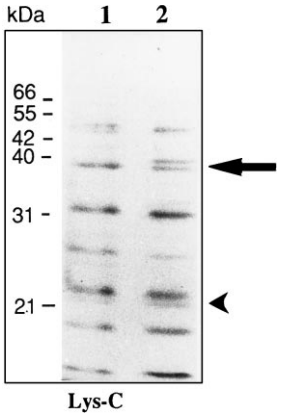
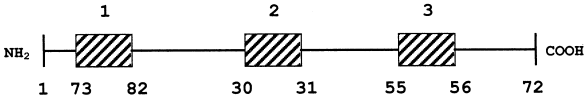


Fig. 2. Comparison of the peptide maps of proteolytic fragments after digestion of purified Mip-90 (lane 1) and hsp-90 (lane 2) with Lys-C endoproteinase. The arrow shows the additional band observed in the hsp-90 proteolytic pattern and the arrowhead points to a band that is enriched in the hsp-90 pattern. The relative molecular weight standards (in kDa) are indicated at the left.

3. Results

In order to establish the identity of Mip-90, we microsequenced fragments of this protein. Purified Mip-90 was partially digested with the lysine endoprotease that cleaves C-terminal lysines in the polypeptide chain. Three of the sequenced fragments permitted an analysis of 8–10 amino acids per fragment. A striking similarity between Mip-90 fragments and the human hsp-90 protein was revealed in this analysis (Fig. 1). The amino acid sequences of Mip-90 peptides matched with sequences located in separate regions of the



DOMAINS	PROTEIN	SEQUENCE	HOMOLOGY (%)
1	**Mip90**	**IDIIPNPQER**	
	Hsp90 β	IDIIPNPQER	100
	Hsp90 α	INLIPNKQDR	60
2	**Mip90**	**SLTND_ED**	
	Hsp90 β	SLTNDWED	100
	Hsp90 α	SLTNDWED	100
3	**Mip90**	**MEE_KA_FE**	
	Hsp90 β	MEESKAKFE	100
	Hsp90 α	QEEKKTKFE	67

Fig. 1. The upper part shows a schematic representation of the positions of the three Mip-90 sequenced fragments (shaded squares) contained in the primary structure of hsp-90. The comparison of Mip-90 sequenced fragments with the corresponding domains on the hsp-90 α- and β-isoforms are shown in the lower part of the figure. The similarities of Mip-90 with each hsp-90 isoform are indicated in terms of percentage homology relative to the respective hsp-90 sequences.

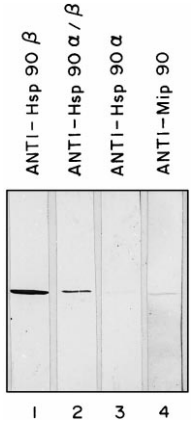


Fig. 3. Immunochemical characterization of a Mip-90, purified by the calmodulin-agarose affinity column, using anti-hsp-90β (lane 1), anti-hsp-90α/β (lane 2), anti-hsp-90α (lane 3) monoclonal antibodies and an anti-Mip-90 polyclonal antibody (lane 4). The calmodulin-purified Mip-90 protein aliquots (5 μg) were loaded and separated in 10% acrylamide electrophoretic gels, electrotransferred onto nitrocellulose strips, and analyzed by Western blots using the different antibodies. Other details are indicated in Section 2.

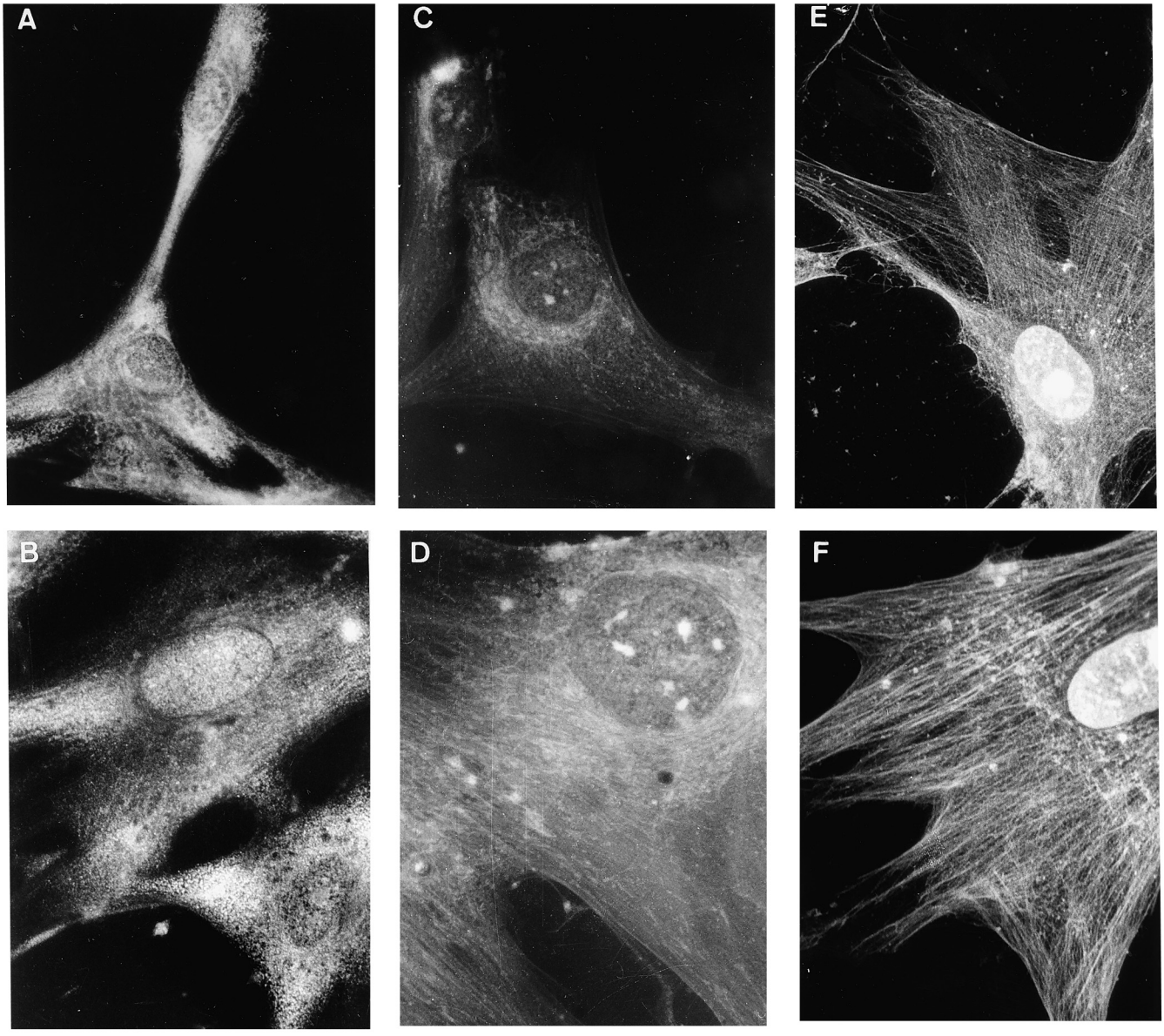


Fig. 4. Subcellular localization of (A,B) hsp-90α (C,D) hsp-90β and (E,F) Mip-90 as analyzed by an immunofluorescence study in subconfluent human fibroblasts cells. Monoclonal antibodies against hsp-90α- and β-isoforms, and a polyclonal antibody against Mip-90 were used. The specificity of the antibodies to hsp-90α and β components were characterized by Nemoto et al. [20,21] Cells were fixed and stained as indicated in Section 2. Bar: 10 μm.

primary structure of human hsp-90, based on hsp-90 sequences reported previously [7,22,23] (Fig. 1, shaded squares), indicating that the similarity was not restricted to a unique domain on hsp-90. This sequence comparison revealed the identity of the three Mip-90 peptides with the β-form of the hsp-90. Meanwhile the percentage of similarity with the α-form of hsp-90 was only 60% and 67%, for the first and the third Mip-90 peptides, respectively (Fig. 1). These peptides are contained in regions of low homology between α- and β-isoforms of hsp-90. Thus, the data obtained from this set of peptides indicate that Mip-90 corresponds to the β-isoform of hsp-90.

Additional studies based on the chromatographic procedures previously used to purify either Mip-90 [6] or hsp-90 [14], namely calmodulin affinity columns and DEAE cellulose and hydroxyapatite columns respectively, revealed that these proteins shared common biochemical features (data not shown), thus confirming their relationship at the protein primary structure level.

The structural relationship between Mip-90 and the purified hsp-90 was further analyzed by examining peptide maps of both proteins using the procedure of Cleveland et al. [17]. In spite of the high degree of similarity between the patterns of proteolytic fragments generated by endoproteinase Lys-C (Fig. 2) we observed some minor differences. Thus, one peptide band was enriched (arrowhead) and another specifically associated to hsp-90 (arrows). Studies using Glu-C endoproteinase also revealed a striking similarity of Mip-90 and hsp-90 patterns. An analysis of Lys-C and Glu-C target sites, using a protein digestion web service from the UCSF Mass Spectrometry Facility (MSF), revealed several differences between the α- and β-isoforms of hsp-90 in the number and

position of the digestion sites. Since the purified hsp-90 contained both the α- and β-isoforms, the differential bands observed in the hsp-90 sample as compared with the Mip-90 pattern, could be a result of the presence of α-hsp-90.

Finally, immunological criteria were very important in order to examine the identity of Mip-90 as the β-isoform of hsp-90. In these studies, samples of Mip-90 eluted from a calmodulin-agarose affinity columns were used to challenge specific monoclonal antibodies against α- or β-isoforms of hsp-90 (Fig. 3). The Western blot analyses ($n=4$) indicate that Mip-90 was recognized by the monoclonal antibody against hsp-90β (Fig. 3, lane 1), as well as by the antibody anti-hsp-90 α/β (Fig. 3, lane 2). A considerably minor reactivity was observed with the anti-hsp-90α antibody (Fig. 3, lane 3). As a control, the presence of Mip-90 was evidenced by using the anti-Mip-90 antibody (Fig. 3, lane 4).

Taken together, the results clearly point toward a structural identity between human Mip-90 and the β-form of heat shock protein hsp-90. In this regard, it was interesting to evaluate the subcellular distribution of hsp-90β and Mip-90. We examined the immunostaining patterns obtained with the monoclonal antibodies against the α- and β-isoforms of hsp-90 as compared with that of Mip-90. Human fibroblasts were used in this study considering that a detailed analysis on Mip-90 organization has been done in these cells [5]. In permeabilized and methanol-fixed cells, hsp-90β displayed a preferential cytoplasmic distribution (Fig. 4C,D). A major fraction of the immunostained protein was associated to filamentous structures and appears to be preferentially localized in a perinuclear network of filaments. These staining features are comparable with those of the cytoplasmic distribution of Mip-90 as assessed using the anti-Mip-90 antibody (Fig. 4E,F). As indicated, previous studies have evidenced that Mip-90 co-localizes with microtubules and actin filaments in these cells [5]. In contrast, hsp-90α presents a more diffuse immunostaining pattern and the protein distributes both in the cytoplasm and in the nucleus (Fig. 4A,B). These results are in agreement with the evidence of the structural similarity between hsp-90β and human Mip-90.

4. Discussion

With the purpose of investigating the structural/functional relationships of the human microtubule-interacting protein Mip-90, the study was focused on the main structural characteristics of this protein. Thus, the sequence of Mip-90 fragments generated by partial proteolysis was analyzed. It was noteworthy to find, after a comparative sequence analysis with protein data banks, a high degree of similarity between this human protein and the heat shock hsp-90. The data revealed a complete identity between the three Mip-90 peptides and the β-isoform of hsp-90. However, sequenced Mip-90 fragments 1 and 3 exhibited only 60–67% similarity with the α-form of hsp-90 [7]. In agreement with this result, fragments 1 and 3 of Mip-90 are contained within hsp-90β regions that show a low level of homology when compared to the hsp-90α sequence. A relevant feature of sequenced Mip-90 fragments is their co-alignment along equivalent sequences of hsp-90β structure, indicating that the criteria of identity of these two proteins is not restricted to a discrete domain conserved on this protein, but rather distributed along N-terminal, C-terminal and intermediate hsp-90 domains.

It was interesting to explore additional structural and immunological properties of Mip-90 to further examine its relationships with α- and β-forms of hsp-90. This analysis appears to be important in the context of the functional relationships between these two proteins. Mip-90 as well as hsp-90 reacted with two different monoclonal antibodies against hsp-90. Since it is unlikely that both antibodies recognize the same epitope on Mip-90, these data suggest that its antigenic relationship with hsp-90 span different domains on the structure of this protein. Moreover, the use of immunological probes that specifically recognize each isoform of hsp-90 [20,21] provides additional demonstration of the identity of Mip-90 as the β-isoform of human hsp-90. The subcellular distribution of hsp-90β seems to reflect its structural identity with Mip-90. Thus, a comparative analysis of hsp-90α- and β-isoforms distribution patterns is of help to clarify some discrepancies arising from previous studies on the immunofluorescence localization of hsp-90 to cytoskeletal filaments [10,24–28].

The data on peptide maps are consistent with structural similarities between purified Mip-90 and hsp90α/β. The presence of different proteolytic fragments in the peptide map of purified hsp-90, when compared with the Mip-90 pattern, may reflect the existence of distinctive sites for protease cleavage on the α-hsp-90 component. Thus, the data allow us to suggest that the minor differences observed in the peptide maps analyses seems to be related to the presence of both isoforms in the pure hsp-90 preparation, while purified Mip-90 corresponds only to the β-isoform of hsp-90. Even though there is limited evidence for posttranslational modifications in hsp-90 [8,29], the possibility that phosphorylations or other modifications in hsp-90 may account for these minor differences cannot be discarded.

In the context of biochemical relationships between Mip-90 and hsp-90 the present studies reveal that hsp-90 under conditions that maintain its native structure interacts with calmodulin in a similar fashion as Mip-90 [5,6]. Moreover, our data suggest that native hsp-90 could be segregated in a β-isoform with the capacity to associate calmodulin and in an α-isoform with a diminished sensitivity to this cell regulator. These differences between hsp-90α and β may be a result of distinct conformational pattern for both components, thus defining differential associations of these isoforms with the calmodulin affinity matrix. As a methodological output of this work, we suggest here the use of calmodulin-agarose chromatography as a tool to purify the β-isoform of hsp-90. Even though we cannot rule out the possibility that this calmodulin-interacting protein corresponds to a subclass of the hsp-90β isoform, to date there is no evidence regarding molecular variants of hsp-90β [8].

Our studies have been focused on the identification and analysis of novel microtubule-interacting proteins with relevant functions on cytoskeletal organization. Within this context Mip-90 was described as a protein that although it is associated with microtubular polymers does not remain associated with microtubules through successive cycles of assembly and disassembly. Thus, previous data indicated that Mip-90 transiently interacts with the tubulin/microtubule system [6]. On the basis of the results reported in this work, and our previous characterization of Mip-90 [5,6], an increasing number of evidence regarding the relationship between hsp-90 and the cytoskeleton [10,24–28,30–32] can be reconciled. Since in the cell, hsp-90 exists as homodimer of β/β- or α/α-isoforms

[23–27], it is reasonable to propose that different isoforms interact with different cell components. Particularly, our results suggest that the association of hsp-90 with microtubules and microfilaments can be re-examined, considering that our data indicate that only the β-isoform of hsp-90 behaves as a cytoskeleton-interacting protein. This possibility would be evaluated in future experimental approaches.

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