A 25-kD Inhibitor of Actin Polymerization Is a Low Molecular Mass Heat Shock Protein

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Abstract. The 25-kD inhibitor of actin polymerization (25-kD IAP), isolated from turkey smooth muscle (Miron, T., M. Wilchek, and B. Geiger. 1988. *Eur. J. Biochem.* 178:543–553.), is shown here to be a low molecular mass heat shock protein (HSP). Direct sequence analysis of the purified protein, as well as cloning and sequencing of the respective cDNA, disclosed a high degree of homology (67% identity, 80% similarity) to the human 27-kD HSP. Southern blot of chicken genomic DNA disclosed one band, suggesting the presence of a single gene, and Northern blot analysis revealed abundant transcript of ~1 kb in gizzard and heart tissues and lower amounts in total 18-d chick embryo RNA and in cultured fibroblasts. Exposure of the latter cells to 45°C resulted in over 15-fold increase in the apparent level of the 25-kD IAP protein, confirming that its expression is regulated by heat shock. Immunofluorescent microscopic localization indicated that after heat treatment, the levels of the 25-kD IAP were markedly increased and the protein was apparently associated with cytoplasmic granules. Heat shock also had a transient, yet prominent, effect on the microfilament system in cultured fibroblasts: stress fibers disintegrated within 10–15 min after incubation at 45°C, yet upon further incubation at the elevated temperature, conspicuous actin bundles were apparently reformed.

THE assembly and modulation of the microfilament network in eukaryotic cells depend not only on the presence of its major building block, actin, but also on the presence and activity of a large variety of actin-associated proteins (Korn, 1982; Pollard and Cooper, 1986; Stossel et al., 1985; Vandekerckhove, 1990). The coordinated action of these components appears to affect a large variety of actin-related cellular processes, including cellular morphogenesis and motility, membrane dynamics, and cell adhesion. One of the microfilament-associated proteins that is involved in the latter process is vinculin (Geiger, 1979). Recent studies have shown that vinculin is associated with the cytoplasmic faces of all types of adherens junctions and may serve as a ubiquitous hallmark for this family of cell contacts (Geiger, 1982). Early attempts to characterize the mode of action of vinculin suggested that it might reduce the low-shear viscosity of F-actin in vitro (Jockusch and Isenberg, 1981; Lin et al., 1982; Wilkins and Lin, 1982). However, further attempts to characterize this actin-binding and modulating activity yielded contradictory results (Evans et al., 1984; Wilkins et al., 1986; Ruhnau and Wegner, 1988), some of which suggested that actin was not directly affected by vinculin itself but rather by other molecule(s) that copurify with it (Evans et al., 1984). Such a "contaminant" was isolated by us and denoted as 25-kD inhibitor of actin polymerization (25-kD IAP)[1] (Miron et al., 1988). This protein was mainly detected in muscular (smooth, cardiac, and skeletal) chick tissues, and hardly detectable in fibroblasts or focal contacts. Moreover, it markedly reduced low-shear viscosity of F-actin and suppressed the rate of polymerization in vitro (Miron et al., 1988). Further studies indicated that the 25-kD IAP does not affect the length of the lag period in the polymerization of gel-filtered actin and thus behaves as a barbed-end capping protein devoid of nucleating activity (Miron, T., M. Wilchek, and B. Geiger, unpublished results). Immunological analysis suggested that the 25-kD IAP is distinct from the HA-1 protein described by Wilkins et al. (1986) (see also Miron et al., 1988). In this paper we present biochemical and molecular genetic evidence indicating that the gizzard 25-kD IAP is a low molecular mass heat shock protein (HSP) homologous to the human 27-kD HSP (Hickey et al., 1986). The expression of 25-kD IAP in cultured fibroblasts increased dramatically after heat shock at 45°C and was detected in cytoplasmic granules. Actin, in the heat-treated cells, underwent reorganization, manifested by conspicuous disassembly of stress fibers after short heat shock (5–15 min), followed by reassembly after a longer exposure (>30 min) at the elevated temperature.

Materials and Methods

Purification of the 25-kD IAP

The 25-kD IAP was extracted from freshly frozen turkey gizzards according to Feramisco and Burridge (1980) with the following slight modifications. (*a*) The frozen muscle (100 g), already trimmed free of fat and connective

1. *Abbreviations used in this paper*: HSP, heat shock protein; IAP, inhibitor of actin polymerization.

tissues, was ground in a cold meat grinder and subjected to homogenization with 0.5 mM PMSF (500 ml) at 4°C. The supernatant was collected (sup-1), and the pellet was subjected to short homogenization with low ionic strength buffer (pH 9; 500 ml) and extracted for 30 min at 37°C. Supernatant of this step (sup-2) was collected. (*b*) The combined supernatants (sup-1 and sup-2) were precipitated with 10 mM $MgCl_2$ and the supernatant was fractionated with ammonium sulfate (20.5 g/100 ml). The precipitate was redissolved and dialyzed as described (Miron et al., 1988). (*c*) Chromatography on DEAE-cellulose and hydroxyapatite was performed according to Miron et al. (1988).

Protein Electroblotting and Microsequencing

Partially purified 25-kD IAP was subjected to 12.5% PAGE in the presence of SDS. Immediately after electrophoresis, the gel was blotted onto polyvinylidene difluoride membrane. Electrophoretic transfer was carried out according to Bauw et al. (1989). The blotted protein was stained for 30 s with 0.1% amido black in a methanol (45%)/acetic acid (7%) solution, destained with distilled water, and air dried. The spot of the 25-kD IAP was cut out, quenched by 0.2% polyvinylpyrrolidone in methanol solution to lower nonspecific adsorption, and was subjected to either trypsin or ASP-N endoprotease (Boehringer-Mannheim GmbH, Mannheim, FRG) digestion in 0.1 M Tris-HCl, pH 8, for 4 h at 37°C. The peptides released from the blot were separated by HPLC on a C4-reversed-phase column (0.46 × 25 cm; Vydac Separations Group, Hesperia, CA) and equilibrated with trifluoroacetic acid (0.1%). Peptides were eluted by a linearly increasing gradient of acetonitrile (1% per min) in 0.1% trifluoroacetic acid. Eluted peptides, detected by UV absorbance at 214 nm, were collected by hand in Eppendorf tubes. The samples were dried in a Speed Vac centrifuge and sequenced by applying the peptide (redissolved in 0.1% trifluoroacetic acid, 30% acetonitrile) onto a Polybrene-coated glass filter. The sequence analysis was carried out with a gas-phase sequenator (model 477A; Applied Biosystems, Inc., Foster City, CA), equipped with an on-line PTH-amino acid analyzer (model 120A; Applied Biosystems, Inc.).

Molecular Genetic Techniques

A chicken gizzard cDNA library in λgt11 bacteriophage was purchased from Clonetech (Palo Alto, CA). The original titer of the library was ~10^9 PFU/ml with 90% recombinants and an average insert size of 1.7 kb. Screening of the library was carried out using polyclonal rabbit antibodies (for the general methodology see Young and Davis, 1988). Inserts were excised from the phage DNA by EcoRI digestion and recloned into Bluescript KS+ vector (Stratagene Cloning Systems, La Jolla, CA). For sequencing, we usually selected clones displaying both orientations to allow for bidirectional single-strand sequencing. Southern and Northern blot analyses were carried out under high stringency conditions (hybridization at 42°C, washing at 68°C) as described (Sambrook et al., 1989).

Actin Localization

Localization of actin within cells was determined by staining with rhodamine-labeled phalloidin (Sigma Chemical Co., St. Louis, MO) after 1 min permeabilization with 0.1% Triton X-100 in 50 mM MES buffer, pH 6.5, and fixation with 3% paraformaldehyde (30 min).

Immunological Methods

Preparation of mAbs to the 25-kD IAP was carried out according to the procedure described in detail by Eshhar (1985). Affinity purification of the mAbs on anti-mouse IgG column and immunofluorescence microscopic localization of the 25-kD IAP were performed according to Miron et al. (1988). Polyclonal antibodies to the 25-kD IAP were prepared in rabbits by repeated injections of the pure protein, emulsified in Complete Freund's Adjuvant (Difco Laboratories, USA). Antibody titers were evaluated by RIA or Western blot analysis (Towbin et al., 1979) after SDS-polyacrylamide electrophoresis on 12% gels (Laemmli et al., 1970).

Heat Stress Conditions

Chicken embryo fibroblasts were prepared from 7-d embryos according to Avnur and Geiger (1981). Cells were grown in Dulbecco's MEM containing 10% FCS on either tissue culture dishes (6 cm) or sterile glass cover slips. For stress conditions, the medium was replaced with preheated medium (45°C): for short heat shock (5–30 min) cells were incubated in a water bath (45°C), while for longer stress (>30 min), cells were maintained in an incubator at 45°C. Conditions for double stress were as described by Collier et al. (1988).

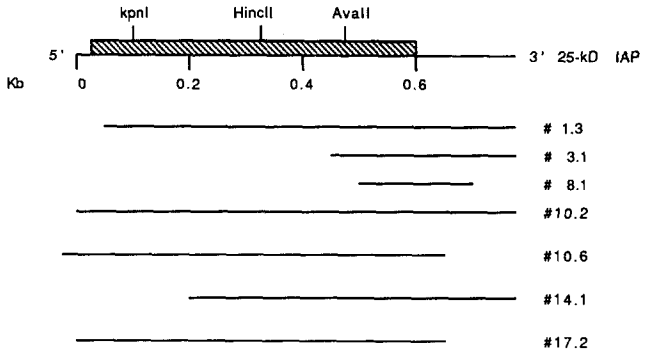

Figure 1. An outline of the seven cDNA clones encoding the 25-kD IAP and their alignment along the respective mRNA (*top*). The box represents the coding region along the mRNA. The location of several restriction sites (as marked) is indicated.

Results

The Primary Structure of the 25-kD IAP

To obtain the complete primary sequence of the 25-kD IAP, we cloned the respective cDNA from chicken gizzard λgt11 expression library. Initial attempts to use the mAbs for library screening were unsuccessful and we, therefore, produced rabbit polyclonal antibodies. One of the clones (3.1, 340 bp), revealed by screening with these antibodies, contained sequences corresponding to those obtained by the direct protein sequencing. The cDNA of this clone was, therefore, used for reprobing the library and revealed additional independent clones, as shown in Fig. 1. Alignment of these clones was achieved by restriction endonuclease mapping and ultimately by complete sequencing.

As shown in Fig. 2, an open reading frame of 579 bp fol-

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1   GGGCGCACCATGGCCGAGCGCCGCGTGCCCTTCACCTTCCTCACCAGCCCCAGCTGGGAG   60
            M  A  E  R  R  V  P  F  T  F  L  T  S  P  S  W  E

61  CCCTTCCGCGACTGGTACCATGGCAGCCGCCTCTTCGACCAGTCCTTCGGGATGCCGCAC  120
    P  F  R  D  W  Y  H  G  S  R  L  F  D  Q  S  F  G  M  P  H

121 ATCCCCGAGGATTGGTACAAGTGGCCCAGCGGCAGCGGCCTGGCCCGGATACTTCCGTCTG  180
    I  P  E  D  W  Y  K  W  P  S  G  S  A  W  P  G  Y  F  R  L

181 CTGCCCAGCGAGAGCGCCCTGCTGCCGGCCCCTGGGTCGCCCTACGGCCGGGCGCTGAGC  240
    L  P  S  E  S  A  L  L  P  A  P  G  S  P  Y  G  R  A  L  S

241 GAGCTGAGCAGCGGCATCTCCGAGATCCGGCAGAGCGCCGACAGCTGGAAGGTCACCCTG  300
    E  L  S  S  G  I  S  E  I  R  Q  S  A  D  S  W  K  V  T  L

301 GACGTCAACCACTTTGCTCCTGAGGAGCTGGTGGTGAAGACTAAGGATAACATCGTGGAG  360
    D  V  N  H  F  A  P  E  E  L  V  V  K  T  K  D  N  I  V  E

361 ATCACCGGCAAACACGAGGAGAAACAGGATGAGCACGGCTTCATCTCCAGGTGCTTCACC  420
    I  T  G  K  H  E  E  K  Q  D  E  H  G  F  I  S  R  C  F  T

421 CGCAAATACACCCTCCCCCCAGGCGTGGAAGCCACAGCCGTGCGGTCCTCGCTGTCCCCC  480
    R  K  Y  T  L  P  P  G  V  E  A  T  A  V  R  S  S  L  S  P

481 GATGGGATGCTGACAGTGGAGGCCCCGCTGCCCAAGCCAGCCATCCAGTCATCCGAGATC  540
    D  G  M  L  T  V  E  A  P  L  P  K  P  A  I  Q  S  S  E  I

541 ACCATCCCCGTCACCGTCGAGGCCAAGAAGGAAGAACCAGCCAAGAAGTAGGAGGAGGCC  600
    T  I  P  V  T  V  E  A  K  K  E  E  P  A  K  K

601 CCGCTGCTGTCCCCTGCCATGCACCCCGCACGCCTGCCCCCAGCTCCCACCCCCCTTTGT  660

661 ATTTTTTATTTGGTACTCACCCATCACCCAGTGCCTTGAATAAACGTGGTGGAAAAGAGA  720

721 AAATTGATGGGAATAAAAGTGGTGGAAGAAAAAAAAAAAACCC  763
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Figure 2. The complete nucleotide and deduced amino acid sequences of the 25-kD IAP, obtained by sequencing of the cDNA clones described above. These sequence data are available from EMBL/GenBank/DDBJ under accession number X59541.

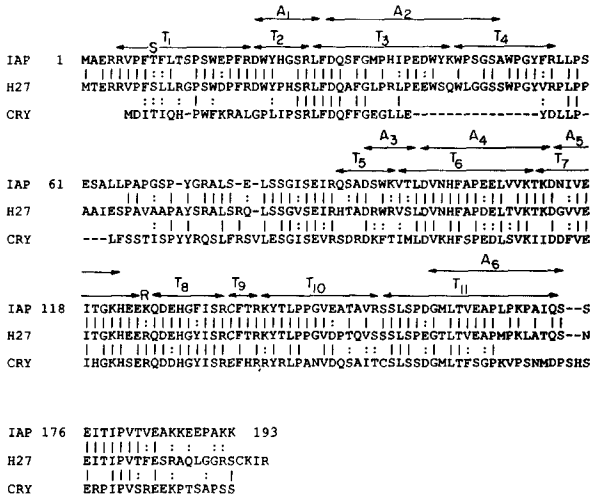

Figure 3. The primary sequence of translated cDNA derived from the chicken 25-kD IAP (*IAP*) is compared to that of low molecular mass human HSP 27 (*H27*) and chicken α-crystallin (*CRY*). The tryptic peptides (T_1–T_{11}) derived from pure turkey gizzard 25-kD IAP, and those produced by Asp-N endoprotease digestion (A1–A7) are indicated by the arrows. Vertical bars and colons indicate amino acid identities and similarities, respectively. Dashes represent gaps introduced to obtain best fit. These sequence data are available from EMBL/GenBank/DDBJ under accession number X59541.

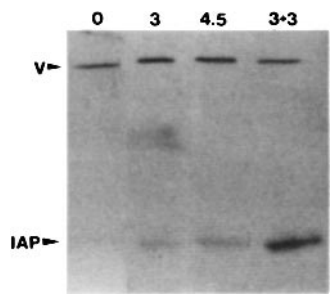

Figure 4. Immunoblot analysis of chicken fibroblasts before and after heat shock, using mAbs to the 25-kD IAP (mAbs mixture) and to vinculin (mAb 11.5). Samples included: untreated fibroblasts maintained at 37°C (0), heat-shocked cells incubated at 45°C for 3 h (3), 4.5 h (4.5), as well as double-shocked cells (3+3). Application of anti-25-kD IAP alone yielded only one band. The labeling with antivinculin was introduced as an internal marker and previous examination indicated that it was not altered after heat shock. The location of the 25-kD IAP (*IAP*) and vinculin (*V*) bands is indicated with arrowheads.

lowing the presumptive initiator AUG was found, encoding a protein of 193 amino acid residues. Analysis of the sequences thus obtained (as well as sequences generated by direct protein sequencing as described below) against the EMBL data base pointed to a high degree of homology (67% identity, 80% similarity) with the human 27-kD HSP (HSP 27) (Hickey et al., 1986). α-Crystallin exhibited lower, yet significant homology to the chicken IAP (44% identity, 60% similarity) and human HSP (Fig. 3).

The deduced sequences were further confirmed by direct sequencing of peptides generated by enzymatic cleavage of the purified 25-kD IAP. As illustrated in Fig. 3, the tryptic (marked T_{1-11}) and ASP-N (marked A_{1-6}) peptides cover a total of 140 amino acid residues. They are compared here with the complete deduced sequences of the 25-kD IAP, HSP 27, and α-crystallin. Comparison of the protein sequences to those obtained by cDNA sequencing indicated that the two were nearly identical except for two conservative substitutions (S-T at position 9 and R-K at position 125). It is not clear whether these represent polymorphic variations or chicken/turkey differences. Comparison of the peptide sequences to those of the human HSP 27 pointed to a high degree of homology (>85% identity within the overlap region) and disclosed a lower homology to α-crystallin (de Jong et al., 1984; Thompson et al., 1987).

The isolated cDNA clones reacted, in a Southern blot analysis with only one band in total genomic chicken DNA, digested with PstI, HindIII, and BamHI restriction endonucleases (not shown), suggesting the presence of a single gene.

Expression of the 25-kD IAP in Cells and Tissues

The notion that the 25-kD IAP is a HSP was directly examined by heat shock of cultured fibroblasts. As previously indicated, these cells normally exhibit low levels of 25-kD IAP (determined by immunoblotting), but when heated to 45°C, especially when double heat-shocked at 45°C (Collier et al., 1988), the apparent levels of the 25-kD IAP increased up to ~15-fold (Fig. 4). This was evident from densitometric scanning of the autoradiograms obtained after immunoblotting. Multiple samples of both the normal and heat-shocked cultures were examined to render the assay quantitative.

Immunofluorescent staining of such fibroblasts showed that while control cells (cultured at 37°C) displayed only faint dotted nuclear staining (the nature of which is not entirely clear), the level of labeling in the cytoplasm after heat shock was very high. At first (0.5–3 h), positively labeled granules appeared in the cytoplasm (Fig. 5), and upon double heat-shock apparent colocalization of the cytoplasmic 25-kD IAP with mitochondria was often noticed (not shown).

Northern blot analysis with total RNA extracted from different chicken tissues indicated that the transcript encoding the 25-kD IAP (~1 kb long) was abundant in gizzard and heart and detected at lower levels in total 18-d embryos or cultured fibroblasts (Fig. 6), in line with previous results obtained at the protein level.

Effect of Heat Shock on the Organization of the Microfilament System

Heat shock also had a transient, yet prominent, effect on the microfilament system. In chick fibroblasts maintained at 37°C, actin is predominantly organized in large stress fibers attached, at their termini, to the cytoplasmic faces of focal contacts (Fig. 7 *A*). After a short (5 min) incubation at 45°C, deterioration of actin stress fibers was noticed (Fig. 7 *B*), which became more extensive upon further incubation (Fig. 7, *C–E*). However, upon longer incubation (3 h) an extensive network of stress fibers reappeared (Fig. 7 *F*).

Discussion

The present study, identifying the 25-kD IAP as an HSP homologous to the human HSP 27 (Hickey et al., 1986), bears some important implications both for the heat shock response (or stress response in general), and for the mechanisms that induce actin modulation in cells.

The heat shock response has been extensively studied in recent years in diverse organisms, from prokaryotes to man

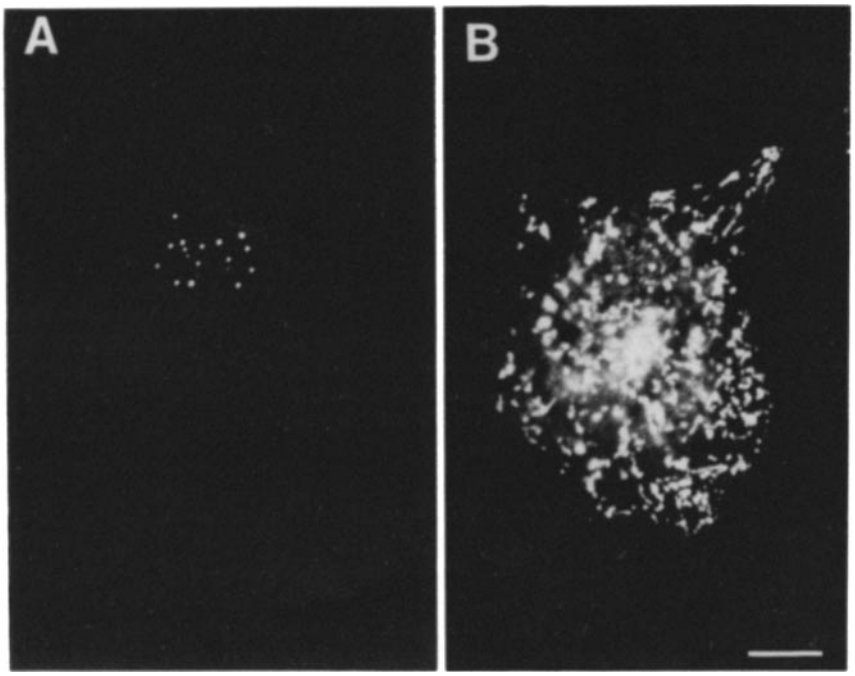

Figure 5. Immunofluorescence labeling of chick embryo fibroblasts maintained at 37°C (*A*), and heat shocked at 45°C for 3 h (*B*) with anti-25-kDa IAP (mixed mAbs). Notice that in the nonshocked controls there is essentially no cytoplasmic labeling and only faint dotted staining is detected in the nucleus. After heat treatment, intense labeling is detected in cytoplasmic granules. Bar, 10 μM.

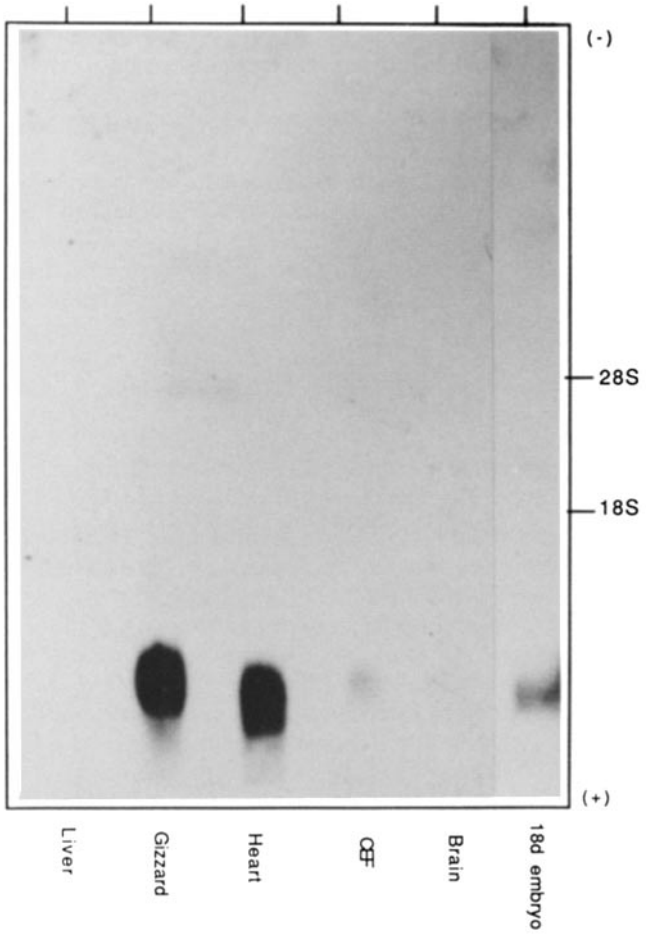

Figure 6. Northern blot analysis of total RNA (30 μg/lane) isolated from several chicken tissues, cultured fibroblasts (*CEF*) and 18-d embryo. The cDNA probe was prepared from clone 10.6, and hybridization and washing were carried out under high stringency conditions.

(Schlesinger et al., 1982). The induced cascade of cellular and molecular events after the exposure to elevated temperature, or to other forms of stress, has been investigated at several levels. At the molecular level, families of HSPs, whose expression is induced or highly augmented after stress treatment, have been identified (Schlesinger et al., 1982; Lindquist, 1986; Schlesinger, 1986; Lindquist and Craig, 1988; Welch et al., 1989). These stress proteins are usually classified into two major subgroups: the relatively high molecular mass (65–110 kD) HSPs and the low molecular mass (15–30 kD) proteins (Lindquist and Craig, 1988). It is generally assumed that these stress-induced proteins enable cells to cope with altered environmental conditions, yet only little is known about their exact functions (Schlesinger, 1986; Susek and Lindquist, 1989). It has nevertheless been argued that some of the HSPs are potent chaperons, displaying a general capacity to interact with a variety of cellular proteins and nucleic acids and possibly modulate their cellular distribution, stability, or reactivity (Ellis and Hemmingsen, 1989; Rothman, 1989). Moreover, it was noted that the various HSPs have been highly conserved throughout evolution, from bacteria to man, suggesting that they play critical and indispensable roles.

The present study indicates that the 25-kD IAP is a member of the low molecular mass HSP sub-family. The major support for this notion is the close sequence homology between the human HSP 27 and the turkey or chick 25-kD IAP, as well as the elevated expression of the latter in fibroblasts after heat shock treatment. Other physico-chemical properties reported for several HSPs, such as the tendency to form oligomeric aggregates (Collier and Schlesinger, 1986; Rossi and Lindquist, 1989), are also observed with the 25-kD IAP (paper in preparation).

At the cellular level, heat response has diverse apparent manifestations (Schlesinger, 1986; Lindquist and Craig, 1988). It has been shown that different cytoskeletal networks undergo remarkable modulation after exposure to heat or to

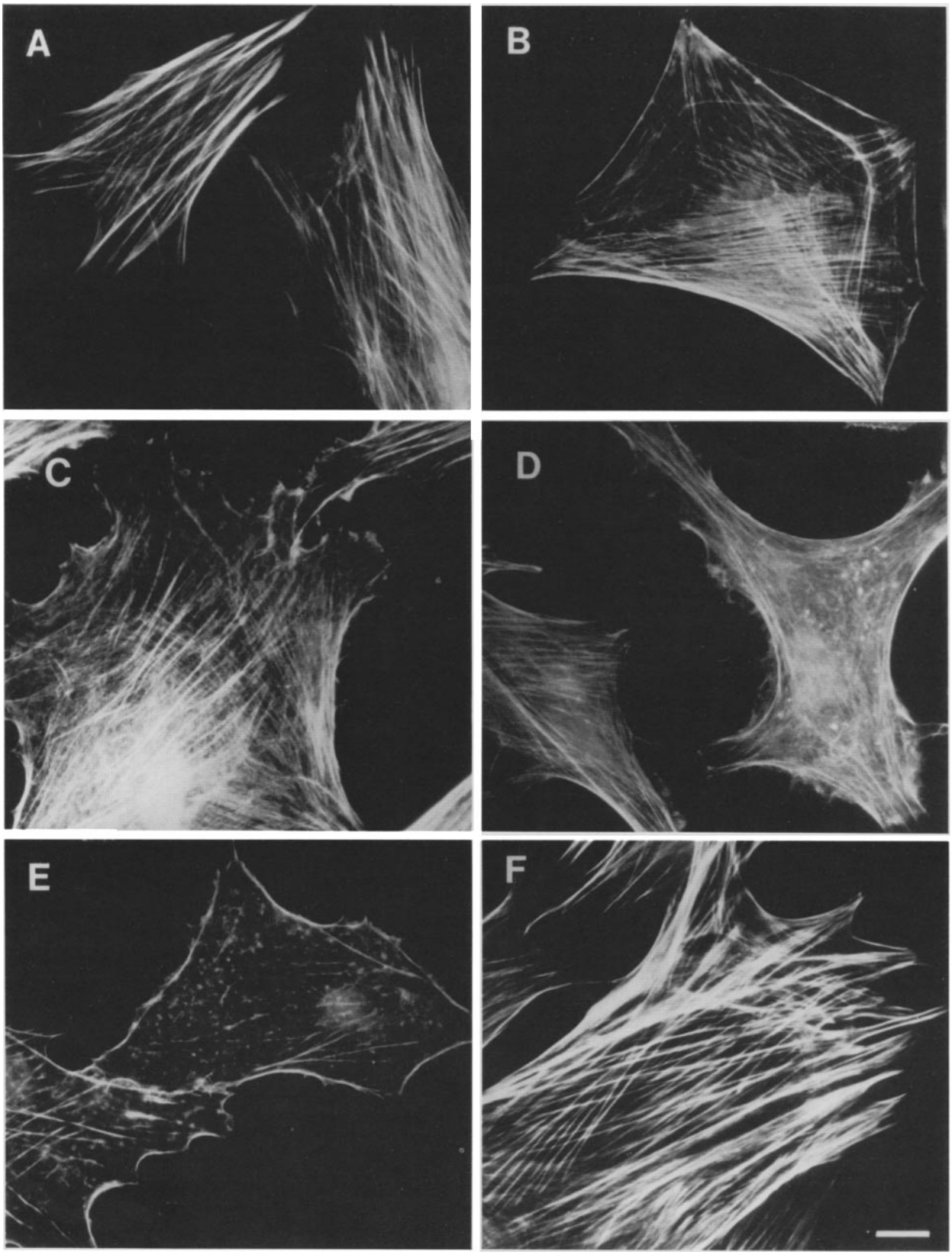

Figure 7. Rhodamine phalloidine staining of actin in chicken embryo fibroblasts cultured at 37°C (*A*) or following exposure to elevated temperature (45°C) for 5 min (*B*), 15 min (*C*), 30 min (*D*), 1 h (*E*), 3 h (*F*). Notice the progressive deterioration of stress fibers which is detectable already after 5 min exposure to 45°C and to the apparent reformation of actin bundles after long exposure. Bar, 10 μM.

other forms of stress. Actin-containing microfilaments were reported to become disorganized (Glass et al., 1985; Van Bergen en Henegouwen and Linnemans, 1987; Shyy et al., 1989), microtubules undergo disassembly (Van Bergen en Henegouwen and Linnemans, 1987; Shyy et al., 1989; Lin, P. S., 1982), and intermediate filaments of the various classes collapse towards the perinuclear area (Biessmann et al., 1982; Collier and Schlesinger, 1986; Shyy et al. 1989; Welch and Suhan, 1985; Welch et al., 1985). Our direct examination of the organization of the microfilament system, after incubation at 45°C, was in line with Shyy et al. (1989), indicating that microfilament bundles undergo transient disassembly followed by a reassembly. It remains to be determined whether the rapid disorganization of actin is directly attributable to the expression of the 25-kD IAP. It is interesting that, in addition to the cytoskeletal reorganization, major changes have been observed also in overall cell shape and in the cytoplasmic disposition of organelles after heat treatment (Collier and Schlesinger, 1986; Shyy et al., 1989). While the particular cellular manifestations of heat shock appear to be quite diverse, many of the induced changes could be affected by reorganization of the cytoskeleton. Several hypotheses have been put forward to account for the heat-induced effects on the various cytoskeletal systems. These include a direct binding of HSPs to the cytoskeleton (Reiter and Penman, 1983; Leicht et al., 1986; Nishida et al., 1986; Ohtsuka et al., 1986) and changes indirectly induced by the arrest in protein synthesis (Tanguay, 1983; Welch and Feramisco, 1985). The results presented in this paper provide a direct indication for a potential link between the induction of a specific HSP and the reorganization of the cytoplasmic matrix.

The exact mechanism by which 25-kD IAP affects actin polymerization was not fully elucidated. However, our previous and current results suggest that the 25-kD IAP acts like an F-actin barbed-end capping protein with little or no effect on actin nucleation. This follows from (*a*) the reduction of the low-shear viscosity of actin filaments (Miron et al., 1988); (*b*) the rise in critical actin concentration under physiological salt conditions; and (*c*) the absence of an effect on the lag time of polymerization. This inhibitory activity is further manifested by the ability of 25-kD IAP to induce disassembly of F-actin, and even of α-actinin crosslinked actin filaments (Miron et al., 1988). It is tempting to speculate that an abrupt increase in the cytoplasmic levels of the 25-kD IAP, after stress induction, might be involved in the disassembly of cytoplasmic actin filaments. This suggestion, however, should be considered with some caution since the heat-induced disassembly of actin bundles, when apparent, is transient (Fig. 7; see also Glass et al., 1985; Shyy et al., 1989), while the presence of the 25-kD IAP persists for many hours. Moreover, the deterioration of actin bundles is observed shortly after heat shock, before an appreciable increase is observed in the level of the 25-kD IAP. We do not have a direct explanation for the apparent recovery of the microfilament system after the heat-induced disassembly, but several possibilities should be considered, including a modification of the 25-kD IAP protein and the appearance of molecules that perturb its interaction with actin or change its physical state. It is also possible that sequestration of the IAP protein within the cytoplasm might prevent it from interacting with actin. These possibilities are currently under investigation.

The immunocytochemical results presented here suggest that the cellular distribution of the 25-kD IAP changes as a function of time after heat shock. Initially, the protein is apparently associated with cytoplasmic aggregates similar to those previously described (Collier et al., 1988; Rossi and Lindquist, 1989) and subsequently it appears to colocalize with the mitochondria. The presence of HSPs (including those of low molecular weight) in mitochondria has been documented for different cell types and its physiological significance discussed (Iida and Yahara, 1985; Nickells and Browder, 1988). It is possible that the effect on actin is exerted only by the soluble protein, before it undergoes major aggregation. This possibility is corroborated by our previous results showing that aggregation of the chick 25-kD IAP is accompanied by loss of its inhibitory activity (Miron et al., 1988).

The authors thank D. Ochert for editorial assistance.

This study was supported by a grant from the Revson Foundation, the Israel Academy of Sciences (B. Geiger), the Forschheimer Center for Molecular Genetics at the Weizmann Institute, and the National Fund for Scientific Research of Belgium (J. Vandekerckhove). K. Vancompernolle is holder of a fellowship of the Institute tot Aanmoediging van het Wetenschappelyk Onderzoek in Nijverheid en Landbouw. We also gratefully acknowledge the allocation of short-term European Molecular Biology Organization fellowships to T. Miron and K. Vancompernolle. B. Geiger holds the E. Neter Chair in Cell and Tumor Biology and M. Wilchek holds the Marc R. Gutwirth Chair in Molecular Biology.

Received for publication 19 October 1990 and in revised form 12 April 1991.

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