Biochemical Analysis of Heat-Resistant Mouse Tumor Cell Strains: a New Member of the HSP70 Family

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A series of heat-resistant mutants selected from a murine tumor cell line, RIF-1, display a markedly increased and stable resistance to heat shock. The mutant cell lines were analyzed for differences that may explain their increased resistance. Membrane lipid analysis showed no change in cholesterol content but an increase in the proportion of saturated fatty acids in the phospholipid fraction. Two-dimensional gel analysis revealed a generally increased constitutive synthesis of several major heat shock proteins (HSP), including HSP90, 68, 60, and 28. In addition, a new protein in the 70-kilodalton region is present in the resistant lines. The new protein has a lower isoelectric point than the constitutive HSP70 does, is only weakly induced by heat shock, and is immunologically cross-reactive with other members of the HSP70 family. After heat shock, the mutants display increases in HSP similar to those seen in the wild-type cells and they develop further transient tolerance to heat. Analysis of these mutants may help in understanding the function of HSP, both in normal growth and after heat shock.

The heat shock proteins (HSP), or stress proteins, are synthesized in response to a variety of stresses apart from heat, including ethanol, arsenite, transition metals, release from anoxia, and mutagens (31). The molecular basis for this transient alteration in gene expression is the subject of intensive investigation. In tissue culture and in mouse tumor systems, the appearance of HSP is accompanied by the development of thermotolerance or transient resistance to subsequent heating. The close temporal matching of the appearance and decay of thermotolerance and HSP both in tissue culture (25, 30, 48) and in vivo (29) has led to the hypothesis that HSP function to protect cells from heat damage. However, an increasing number of examples of thermotolerance in the apparent absence of elevated levels of HSP (11, 20, 41, 50) has raised some doubts about this proposed function. At the very least, these results suggest that cells have alternative methods for developing thermotolerance that do not involve the HSP.

One possible alternative mechanism that has received considerable attention is an alteration of the cellular membranes to a more thermostable form (7). Heat increases the fluidity of membranes (28), leading to loss of their selective permeability and possibly to cell death. The importance of the plasma membrane in heat damage is indicated in the synergistic interaction of the membrane-active drug, amphotericin B, with hyperthermia (17) and the enhanced uptake of drugs such as Adriamycin in cells held at elevated temperatures (43 to 45°C) (43). An aggregation of membrane proteins in the plasma membrane occurs immediately after heat shock in cells destined to die (42). Several integral membrane proteins, such as the ouabain-sensitive Na⁺, K⁺-ATPase (1, 8), the Na⁺-dependent amino acid transport protein (34), the epidermal growth factor receptor (35), and the insulin receptor (9) all show diminished activity after hyperthermic treatments. In thermotolerant cells, ouabain-sensitive K⁺ transport (1) and insulin-binding activity (9) are protected from heat damage.

Our approach to exploring the ways in which cells can increase their resistance to heat has been to select heat-resistant mutants and look for changes in their lipid and protein composition that may be responsible for the development of the heat resistance. In a previous paper (19), we described the selection and heat-survival characteristics of several clones of heat-resistant cells selected from a murine radiation-induced fibrosarcoma (RIF-1) by cycles of heating at 45°C followed by regrowth of survivors at 37°C. No chemical mutagens were used during the selection procedure. The thermoresistant (TR) clones exhibit much greater resistance to hyperthermic shock at all temperatures studied than the wild-type cells do. However, they are still able to express a comparable amount of thermotolerance after exposure to an isosurvival heat dose. (A heat dose of 10 min at 45°C causes approximately 50% kill in the RIF-1 parent and induces close to a maximal level of thermotolerance. To achieve 50% kill in the TR clone, a heat dose of 30 min at 45°C must be given, and the level of thermotolerance is similar to that of RIF-1 when measured as a ratio of the slopes of the heat survival curves [19].)

There have been previous reports on the selection of heat-resistant mutants (4, 10, 21, 22, 27), but the RIF-1 mutants described here have several unique features. They display more heat resistance than any others described; they have a morphology and growth characteristics similar to those of the parent line and can grow with comparable growth rates in vivo as solid tumors in C3H mice. In addition, they show several interesting and unique changes in the constitutive pattern of HSP, which may give us an opportunity to establish the physiological function of HSP both before and after heat shock.

In this study, we present data on the lipid composition of the cell membranes and on changes in the heat shock protein composition of the TR lines. In particular, we report the appearance of a new species of HSP70 that is constitutively present in the TR lines.

MATERIALS AND METHODS

Cells. The RIF-1 cells were derived from a radiation-induced fibrosarcoma previously described by Twentyman et al. (49). The cells grow both in vitro and in vivo in C3H mice. In tissue culture, the cells are maintained as monolay-

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ers in RPMI 1640 containing 15% fetal calf serum, 200 mg of streptomycin sulfate per liter, and 190,000 U of penicillin G potassium per liter at 37°C in an atmosphere of 5% CO2-95% air. The procedure used to isolate the thermoresistant cells has been described earlier (19).

**Cell cycle distribution.** Cells in early to mid-log phase of growth were trypsinized, centrifuged, and suspended in an aqueous solution containing 25% ethanol, 15 mM MgCl2, and 100 μg of mithramycin (Pfizer Inc., New York, N.Y.) per ml at a final cell concentration of 0.5 × 106/ml (13). DNA content and cell cycle distribution were measured by flow cytometry by using an argon laser at a wavelength of 457.9 nm.

**Growth curves.** The growth rates of the parent and TR clones were compared over a 10-day period at either 37 or 40.5°C. Exponential-phase cells, 1.5 × 105, were inoculated into 25-cm2 tissue culture flasks. Each day, beginning on day 3, cells were detached into a hemacytometer.

**Hyperthermia treatment.** Cells were heated as monolayers in exponential phase in specially designed incubators that were temperature controlled to ±0.1°C, in an atmosphere of 5% CO2-95% air. The pH was maintained between 7.2 and 7.4 at all times.

**Lipid and protein analysis.** The preparation of the total lipid fraction, the separation of the phospholipid fraction, and the measurement of cholesterol and phospholipid levels have been described earlier (2). Fatty acid analysis of the phospholipid fraction was performed as described elsewhere (6). Protein concentration was measured by the method of Lowry et al. (33).

**Rate of protein synthesis.** The effect of heat on the rate of protein synthesis was determined by the extent of uptake of 3H-labeled amino acids over a 10-min period at 37°C immediately after the hyperthermic exposure. The details of the procedure have been published elsewhere (3).

**Gel electrophoresis.** To analyze the constitutive pattern of proteins synthesized by the parent line and TR clones, exponential-phase cells were incubated at 37°C for 2 days in regular RPMI 1640 containing 15% fetal calf serum and either 2 μCi of [35S]methionine (Amersham Corp., Arlington Heights, Ill.; specific activity, >1,000 Ci/m mole) per ml or 5 μCi of tritiated amino acid mix (ICN Pharmaceuticals Inc., Irvine, Calif.; specific activity, 184 mCi/mg) per ml. To follow the induction of proteins by heat shock, cells were incubated in either 10 μCi of [35S]methionine per ml in methionine-free RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) containing 15% dialyzed fetal calf serum or 20 μCi of tritiated amino acid mix per ml for 6 to 8 h after treatment.

Proteins were analyzed by sodium dodecyl sulfate gel electrophoresis. For one-dimensional analysis, proteins were separated on 13% acrylamide by the Laemmli procedure (24) as described earlier (4). For two-dimensional (2D) analysis, proteins were first subjected to isoelectric focusing in a 3-mm-diameter tube gel in 3.5% acrylamide prior to molecular weight separation on a 10 or 13% sodium dodecyl sulfate acrylamide slab gel (14). The gels were stained with Coomassie blue, destained, and dried, and radiographs were prepared by using Kodak XAR-2 film. Tritium-labeled gels were treated with En3Hance (Du Pont, NEN Research Products, Boston, Mass.) prior to drying. Proteins on 2D gels were quantitated by radioscanning the gels on a proportional gas-flow counter (Automated Microbiology Systems, San Diego, Calif.).

**Western blotting (immunoblotting).** Immediately after electrophoresis, the proteins in a gel were transferred to nitrocellulose paper (Hoefer, San Francisco, Calif.) by electroblotting (E. C. Apparatus, St. Petersburg, Fla.). The nitrocellulose paper was probed with a mouse monoclonal antibody (N6 F3-5) raised against HeLa HSP70. The antibody was provided by W. Welch, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. The binding of the monoclonal antibody was detected by incubation of the paper with an alkaline phosphatase-linked goat anti-mouse immunoglobulin G (kpl Laboratories, Gaithersburg, Md.).

**Measurement of levels of HSP70.** An enzyme-linked immunosorbent assay was developed to quantitate the levels of HSP70 in cell homogenates. The details of the assay are to be published elsewhere. Briefly, the cells were boiled in a small volume of sodium dodecyl sulfate gel sample buffer (24), diluted with phosphate-buffered saline, sonicated, and boiled again. This essentially solubilized the cells. Samples containing known amounts of protein were placed in the wells of a 96-well dish prior to addition of the N6 F3-5 monoclonal antibody against HSP70. The amounts of bound antibody were measured by the rate of reaction of horseradish peroxidase covalently linked to a goat anti-mouse immunoglobulin G which was added to the wells. An alternate method of preparation of the cell lysate that resulted in analysis of native proteins gave similar results.

**RESULTS**

From the 20 TR clones isolated, 4 were chosen for biochemical analysis. The criteria for this selection and the demonstration of a high degree of heat resistance have been presented elsewhere (19). Examination by light microscopy revealed a morphology similar to that of the parent RIF-1 cells. The growth rate at 37 and 40.5°C was compared for the wild-type cells and the TR clones. All lines had a similar doubling time at 37°C, and at 40.5°C, all failed to proliferate. This is shown in Fig. 1 for the TR5 clone. Thus the resistance seen to brief exposures at 42 to 46°C did not permit the TR clones to grow at mildly elevated temperatures. When mithramycin-stained cells were analyzed by flow cytometry, similar DNA histograms were obtained in the parent and TR clones (data not shown). The TR cells retained the small subpopulation of tetraploid cells that is characteristic of RIF-1 cells (49). This is a curious finding since the TR cells have been cloned; however, Rowley et al. (44) reported that in diploid RIF-1 cells separated from tetraploid cells by centrifugal elutriation, tetraploid contamination increased to 35% by 40 days after separation. The regrowth of tetraploid cells could not be explained by differences in plating efficiency or doubling time.

**Membrane composition.** The cholesterol content of TR4, TR5, and TR10 cells was similar to that found in the parent line, both when expressed on a cell protein basis and when expressed as a molar ratio to total phospholipids of the cell (data not shown). Similar cholesterol levels were seen when expressed on a per cell basis. In addition, the total phospholipid content did not change significantly when the cells became heat resistant.

In contrast, marked changes were observed in the fatty acid composition of membrane phospholipids (Table 1). The major changes were an increase in palmitic and oleic acids and a decline in the levels of arachidonic acid. The net result was a decrease in the unsaturation index from 4.5 in the RIF-1 cells to 2.8 in TR cells.

**Protein composition.** Protein synthesis is a very heat-sensitive process (15, 18, 23, 36). We measured the effect of heating at 43°C on the rate of protein synthesis in the
thermoresistant clones. The results for TR, cells show that protein synthesis is more heat stable, retaining 50% activity after 10 min at 43°C compared with 20% residual activity in RIF-1 cells (Fig. 2). Similar results were obtained for TR, cells (data not shown).

Protein composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A radiograph of [35S]methionine-labeled proteins from RIF-1 and four TR lines separated by one-dimensional gel electrophoresis is shown in Fig. 3. Cells were either incubated for 48 h in a low concentration of the radiolabel to uniformly label all cellular proteins or were labeled for 8 h after heat shock to demonstrate the pattern of heat-induced proteins. In RIF-1 cells subjected to heat shock (Fig. 3, lane 2), the synthesis of at least three proteins, including those at 110, 89, and 70 kilodaltons (kDa) was increased, and a new band at 68 kDa was expressed. In addition, minor induction of a protein at

![Graph showing growth curves of RIF-1 and TR, at 37 and 40.5°C.](http://mcb.asm.org/)

**FIG. 1.** Growth curves of RIF-1 and TR, at 37 and 40.5°C. ■, RIF-1 at 37°C; □, RIF-1 at 40.5°C; ●, TR, at 37°C; ○, TR, at 40.5°C.

![Graph showing rate of protein synthesis at 43°C.](http://mcb.asm.org/)

**FIG. 2.** The rate of protein synthesis in RIF-1 (□) and TR, (■) measured immediately after various times of heating at 43°C.

![Radiograph of acrylamide gel of proteins from RIF-1 (WT) and heat-resistant clones.](http://mcb.asm.org/)

**FIG. 3.** Radiograph of a 15% acrylamide gel of proteins from RIF-1 (WT) and heat-resistant clones 1, 4, 5, and 10. The cells were either unheated (C) or were heated (H) for 10 min at 45°C (RIF-1) or 20 min at 45°C (TR clones). The markers down the lefthand side show molecular weight standards. The bars on the righthand side denote the positions of the major heat shock proteins.

### TABLE 1. Fatty acid analysis of phospholipids from RIF-1 and TR,

<table>
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<tr>
<th>Fatty acid</th>
<th>% Total phospholipid content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RIF-1</td>
</tr>
<tr>
<td>C16:0</td>
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</tr>
<tr>
<td>Unknown</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>C18:2</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>C16:1</td>
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<tr>
<td>C18:2 cis 9,12</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>C18:1</td>
<td>13.2 ± 1.7</td>
</tr>
<tr>
<td>C18:1 tr6/tr9/cis11</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>C18:0</td>
<td>23.2 ± 0.9</td>
</tr>
<tr>
<td>C20:4 cis 5,8,11,14</td>
<td>18.8 ± 2.2</td>
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<tr>
<td>C20:4 cis 6,9,12,15</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>C22:6</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>C22:4</td>
<td>4.9 ± 0.7</td>
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<tr>
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</tr>
<tr>
<td>Unsat* index*</td>
<td>4.5 ± 0.4</td>
</tr>
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* The data show the means and standard deviations for three samples. Similar data were obtained in a separate experiment.

* * (percent unsaturated fatty acids x number of double bonds)/percent saturated fatty acids.
60 kDa was occasionally seen. No major differences in the constitutive protein composition between RIF-1 and the TR clones were revealed by this process. Quantitation of the constitutive levels of the HSP by scanning densitometry of several gels prepared from \[^{35}S\]methionine-labeled proteins was performed. In two or three separate analyses of three of the TR strains, no major changes in the levels of the HSP were observed, except possibly a hint of an increase in HSP89 and 68/70 in TR\(_c\) (data not shown).

However, when the level of HSP68/70 was measured with an enzyme-linked immunosorbent assay, different results were obtained. The monoclonal antibody recognized both the constitutive HSP70 (Hsc70) and the inducible 68 (Hsp68) in RIF cells, as confirmed by 2D Western blotting (see Fig. 7). The TR clones contained 2 to 4 times more constitutive HSP70 than the parent line did. Following heat shock (10 min at 45°C) and incubation at 37°C, HSP70 levels increased in both the wild-type and mutant cells (manuscript in preparation).

2D gel electrophoresis (isoelectric focusing followed by molecular weight separation) revealed the presence of several changes in the protein composition of the TR\(_c\) line (Fig. 4) compared with the wild-type line. Some of the changes were the same as those seen in heat-shocked wild-type cells. In RIF-1, heat shock induced a second component of Hsp89, with a slightly lower molecular weight (upper arrow on Fig. 4B), and the proteins at 68 kDa (underlined). Hsc70 was only weakly heat inducible (lower arrow). These changes are present in TR\(_c\) in the absence of heat shock (Fig. 4C). The 60-kDa protein (circled) was not increased by heat shock in this experiment (Fig. 4B) but was present in increased amounts in unheated TR\(_c\) (Fig. 4C). The major change in TR\(_c\), however, was the presence of a new protein, with a subunit molecular weight of 71 kDa (indicated by the arrowhead), which resolved from Hsc70 by having a slightly lower isoelectric point. The new protein was present in levels similar to Hsc70 and was present in all three of the TR lines that have been analyzed by 2D electrophoresis. The basic protein at approximately 45 kDa was present in all samples (Fig. 5), but because it is near the edge of the gel, it is sometimes trimmed during photography. In Fig. 4, it is visible only in panel B. It did not change after heat shock. In summary, the TR\(_c\) cells (and clones 4 and 10) showed a heat shock response similar to that seen in the wild-type cells but contained higher constitutive levels of several HSP.

The low-molecular-weight HSP are not well visualized by \[^{35}S\]methionine labeling, since they are methionine-poor proteins. However, on fluorographs from cells incubated with \(^{3}H\)-labeled amino acids, a group of four proteins at 28 kDa was prominent after heat shock in both RIF-1 and TR\(_c\) cells (labeled a, b, c, and d). Protein c was constitutively present in RIF-1 and TR\(_c\), and did not respond to heat shock. The three proteins that are heat inducible in RIF-1 (proteins

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FIG. 4. 2D gel analysis of \[^{35}S\]methionine-labeled proteins from RIF-1 and TR\(_c\), before and after heat shock. Proteins were separated on 10% acrylamide after isoelectric focusing. (A) Unheated RIF-1; (B) RIF-1 after 10 min at 45°C; (C) unheated TR\(_c\); (D) TR\(_c\) after 20 min at 45°C. The upper arrow points to HSP89; the lower arrow points to Hsc70. The arrowhead marks the new 71-kDa protein in the TR lines. The underlined proteins are the three members of the Hsp68 group. The encircled protein is HSP60. A. actin.
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isoelectric focusing. After by b, and d) approximately was a 20-min level of Hsp90, increases of 40.5°C have also from the immunostaining.

amounts of Hsp90, Hsc70, and Hsp90. TR₄ cells contained approximately equal amounts of Hsc70 and the new 71-kDa protein (1%). Compared with wild-type cells, there were increased levels of Hsp90, Hsc70, Hsp68, and Hsp60. After a 20-min 45°C heat shock in TR₄ cells, the increase in Hsp68 was again the major response, shifting from 0.4 to 3.4%. The smaller response of Hsp68 in TR₄ cells was probably due to the lower toxicity of 20 min of heating, compared with 10 min in the RIF-1 cells (19).

To determine whether the new protein at 71 kDa was a member of the HSP70 family, Western blots of 2D gels were immunostained with the monoclonal antibody against HSP70. In unheated RIF-1 cells, only one spot, at 70 kDa, was evident on the gels (Fig. 7A). After heat shock, the heat-inducible Hsp68 was also present (Fig. 7B). In TR₄ cells (and in TR₅ cells; not shown), the new protein at 71 kDa and Hsp68 were stained, in addition to Hsc70 (Fig. 7C). In heated TR₄ cells, the main change was an increase in the amount of Hsp68 (Fig. 7D). The identification of these immunostained proteins as HSP70 proteins was confirmed by Western blotting of radioactive gels and matching of the position of the HSP70 proteins on radiographs from the blots with the immunostaining. The same staining pattern was obtained with a different anti-HSP70 antibody (N27 F3-4). also from W. Welch.

DISCUSSION

The TR lines isolated from the RIF-1 cells display a marked increase in heat resistance (19) and several differences at the molecular level as well. However, their morphology, DNA content, and growth rate at both 37 and 40.5°C have not been altered.

The heat-resistant phenotype is very stable. One flask of TR₄ cells was subcultured continuously for 71 passages over a period of 10 months without loss of heat resistance or change in protein composition when compared with regular TR₅ cells. For all experiments presented here, the cells used were between passages 1 and 4.

In the membranes, the composition of the phospholipid fatty acids of clone 5 is different in several components. The net effect is an increase in the overall level of saturation of the fatty acids. The notable changes are an increase in the relative amounts of palmitic and oleic acids and a decrease in arachidonic acid. This latter change possibly has great significance, in view of the role of arachidonic acid as a precursor of the prostaglandins, leukotrienes, and thromboxanes. Arachidonic acid is released from membrane phospholipids by phospholipase A₂, which has been shown recently to be activated by heat (Calderwood, unpublished data). Also, a higher degree of saturation of the fatty acids in the membranes of TR cells implies a less-fluid membrane that may be expected to be more heat stable. The other heat-resistant clones have not been checked for similar changes in fatty acid composition, but in other respects, all the clones have been shown to respond in the same way. The lack of change in the cholesterol content is in marked contrast to the results found with heat-resistant mutants from B16 melanoma cells, where there is a progressive decrease in cholesterol content (and corresponding increase in membrane fluidity) with increasing heat resistance (6).

Protein synthesis is protected from heat damage in the heat-resistant lines. This has been shown previously in other heat-resistant mutants (5, 26) and in cells made thermostoler-ant by prior heat exposure (18, 26, 38, 40, 45).

The 2D gels revealed major changes in the HSP of the heat-resistant lines. The one-dimensional gel (Fig. 3) is included as a warning that it is possible to miss important changes in cell protein composition. The measurement of elevated levels of the HSP70 family in the TR lines by immunoassay led us to believe that we should have seen

FIG. 5. 2D gels of tritium-labeled proteins from RIF-1 and TR₅ before and after heat shock. Proteins were separated on 13% acrylamide after isoelectric focusing. The heat treatment was 10 and 20 min, respectively, at 45°C for RIF-1 and TR₅ cells. This treatment was followed by an 8-h incubation at 37°C. The enclosed area of the RIF-1 gel is the only region displayed for the other three samples. The numbers on the lefthand side are molecular weight markers and the letters a through d mark proteins in the 28-kDa region. The letter H refers to heated samples.
greater amounts of HSP70 on the gels, but it was not until 2D gels were run that the HSP70 increase and that of the other HSP became obvious.

There is good agreement between the analysis of HSP70 levels by 2D gel analysis and the enzyme-linked immunosorbent assay. For example, in comparing the constitutive levels of all members of the HSP70 family, the ratios of levels in TR4 compared with wild-type cells are 2.1 for the enzyme-linked immunosorbent assay and 2.3 for the radio-scanning. The ratios of levels in heated cells compared with control RIF-1 cells are 8.2 and 9.3, respectively.

TR cells reveal increased constitutive synthesis of several HSP or constitutive expression of some that are present only after heat in the wild-type cells. Of particular interest is the constitutive expression of the normally heat-inducible member of the HSP70 family, Hsp68. This protein is also expressed constitutively in human cell lines (51). Human cells display a far greater resistance to heat shock than rodent lines do (manuscript in preparation), and hence Hsp68 may be important in conferring the heat-resistant phenotype. In addition, three isoforms of HSP28 that are heat inducible in wild-type cells are constitutively expressed in the thermore-

sistant lines. HSP28 is the only HSP that is overexpressed in heat-resistant Chinese hamster lung cells isolated after mutagenesis and a single heat shock (12), and the authors speculate that HSP28 is linked to the resistant phenotype.

Recently, a heat shock transcription factor has been described in Drosophila sp., yeast and human cells (39, 47, 52). In Drosophila sp., the heat shock transcription factor binds to the heat shock consensus element after heat shock stimulation and initiates transcription of heat shock genes. The protein is present in cells in an inactive state and is activated, probably by phosphorylation, after heat shock (47). It is possible that the heat shock transcription factor is, at least partially, in a permanently activated state in the TR clones to cause a general increase in expression of the HSP.

In addition to the general increases in HSP levels, there is a new 71-kDa protein present in the TR lines. It has been identified as a member of the HSP70 family by its cross-reactivity to antibodies raised against purified HSP70. The protein does not correspond to any of the HSP70 proteins normally seen in the RIF-1 cells but is occasionally seen in 2D gels of other mammalian cells (51). An analysis of the protein composition of the testes of C. H mice showed also that it is not the developmentally regulated HSP70 gene that is seen in high levels in the testes (unpublished data). The tests protein has a higher isoelectric point than the Hsc70, while the new protein in the TR cells has a lower isoelectric point. The appearance of a new 70-kDa protein in colchicine-resistant mutants of the Chinese hamster ovary line, PdK116, has been reported by Gupta and Gupta (16). However, their new protein also has a higher isoelectric point than the neighboring 70-kDa protein, which has similar migration characteristics on 2D gels to Hsc70, but has not been reported as a member of the HSP70 family.

Several possibilities exist to explain the appearance of this protein in TR cells. It may be a posttranslational modification of either Hsc70 or Hsp68. This possibility is currently being tested by in vitro translation of RNA isolated from

FIG. 6. Quantitation of levels of individual proteins by 2D gel radioscanning. Cell samples were incubated in [35S]methionine for 2 days before and for 6 h after heat shock (where appropriate). The amount of each protein is expressed as the percentage of counts present in the spot to total counts on the gel. Hsp28 cannot be quantitated in this way because of the low-energy photons emitted by tritium atoms. Solid bars, unheated cells; open bars, cells heated for 10 min (RIF-1) or 20 min (TR4) at 45°C.

FIG. 7. Western blots of proteins from RIF-1 and TR4 cells separated by 2D electrophoresis and probed with a monoclonal antibody against HSP70. Unheated RIF-1 cells (panel A); RIF-1 cells given 10 min at 45°C and then 7 h at 37°C (panel B); unheated TR4 cells (panel C); TR4 cells given 20 min at 45°C and then 7 h at 37°C (panel D). The arrow points to the constitutive Hsc70, the upward-pointing arrowhead indicates the heat-inducible Hsp68 and the downward-pointing arrowhead indicates the new 70-kDa protein.
both the parent and TR cells. Alternatively, it may result from the transcription of a new HSP70 gene not normally transcribed. Finally, there may have been a point mutation in a HSP70 gene during the selection process or the activation of a pseudogene. In any case, it would represent the first time that either a posttranslational modification or a natural mutation in the HSP70 gene has been described in mammalian cells.

In preliminary experiments performed in collaboration with Larry Moran in Toronto, RNA preparations from non-heat shocked RIF-1 and TR, cells have been probed by Northern (RNA) analysis with four cDNA probes believed to represent all the HSP70 genes in mouse cells (32). Only the cognate Hsc70 RNA was present in the parent line, whereas the TR cells contained a low level of Hsp68 message, in addition to a similar amount of the Hsc70 RNA. There was no mRNA corresponding to either the testes-specific gene or GRP78. These results imply either posttranslational modification of the Hsc70 protein or the expression of a new HSP70 gene.

There have been previous reports on the isolation and characterization of heat-resistant cell lines selected by using a variety of protocols, some of which involved mutagenizing agents. The point of interest is that, in these studies, no consistent changes in either membrane or protein composition have been observed. The early reports describing the isolation of heat-resistant mutants (10, 21, 22, 45) did not relate the changes in heat response to any molecular changes within the cell. The first analysis of HSP was in heat-resistant cells derived within the cell. The initial analysis of HSP was in heat-resistant cells derived from Chinese hamster ovary cells (27), where an increase in Hsc70 was found. In contrast, heat-resistant variants of B16 melanoma cells (4) revealed no changes in the constitutive pattern of heat shock proteins, but a strong reverse correlation was found between the degree of heat resistance and the cholesterol content of the cells (6).

The reason for selecting heat-resistant mutants was to explore mechanisms of heat resistance in mammalian cells. Because both modifications in membrane lipids and in proteins have been proposed to explain the acquisition of heat resistance, we looked for, and found, changes in both aspects. The acquired heat resistance may be explained by the increased membrane stability to elevated temperatures offered by the increase in saturation of the membrane phospholipid fatty acids. Alternatively, the reduced level of arachidonic acid in the TR lines may have significance, in view of the activation of phospholipase A2 and hence release of arachidonic acid in heated cells.

The acquired heat resistance may also be explained by the elevated levels of one or more of the HSP in the TR cells. Their presence may lead to stabilization of heat-sensitive proteins and organelles in cells, as postulated by Minton et al. (37). Alternatively, the resistance may be due to the presence of the new HSP70 protein, which constitutes 1% of total cell protein in TR cells. Finally, the resistance may not involve any of the above-mentioned changes and may be due to another, as yet undetected, change in these cells.

ACKNOWLEDGMENTS

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LITERATURE CITED