Proteomic Analysis of Protein Phosphorylations in Heat Shock Response and Thermotolerance*

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Heat shock (HS) induces a wide variety of biological processes, including inhibition of protein synthesis, elevated expression of heat shock proteins, induction of thermotolerance, and apoptotic cell death in a dose-dependent manner. We compared phosphorylated proteins in heat-shocked and thermotolerant cells using proteome analysis. After HS treatment of control RIF-1 and their thermotolerant derivatives, TR-RIF-1 cells, cellular proteins were separated by two-dimensional gel electrophoresis and the phosphorylated proteins were detected with the anti-phosphotyrosine antibodies. We found that 93 proteins showed significant changes in phosphorylation between control and thermotolerant cells as a function of recovery time after HS; we identified 81 of these proteins with peptide mass fingerprinting using MALDI-TOF MS after in-gel trypsin digestion. These phosphorylated proteins exhibit various cellular functions, including chaperones, ion channels, signaling molecules, in transcription and translation processes, in amino acid biosynthesis, oxidoreduction, energy metabolism, and cell motility or structure, suggesting that HS turns on the various signaling pathways by activating protein-tyrosine kinases (PTKs). Of these, 20 proteins were previously identified phosphorylated proteins and 64 were newly identified. These proteins can be grouped into three families: 1) proteins highly phosphorylated in TR-RIF-1 cells at basal level and phosphorylated more significantly by HS in RIF-1 than TR-RIF-1; 2) proteins highly phosphorylated in control RIF-1 cells at basal level and phosphorylated more easily by HS in TR-RIF-1 than in RIF-1 cells; and 3) proteins with a similar basal phosphorylation level in both RIF-1 and TR-RIF-1 cells and responding to HS similarly in both cells. Most of the phosphorylated proteins are presumably involved in HS signaling in different ways, with the first and second families of proteins influencing thermotolerance. The possible tyrosine phosphorylation sites, the possible PTKs phosphorylating these proteins, and the proteins binding to these phosphorylated sites were predicted by the Netphos, ScanProsite, and Scansite programs. These results suggest that HS can activate various PTKs and HS responses can be regulated by phosphorylations of proteins having various functions.

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Heat shock responses are well conserved phenomena through evolution. Modest elevations of temperature induce apoptotic cell death. A common feature of the heat shock response is that an initial, nonlethal heat shock provides a transient resistance against subsequent lethal heat shock. This phenomenon is called thermotolerance. Thermotolerant cells induce the overexpression of a family of heat shock proteins (Hsps)§ and are thereby protected from cell death caused by various stresses. This suggests that the chaperonic function of Hsps is associated with the development of thermotolerance. However, the details of the molecular events underlying heat shock responses are not well defined.

Heat shock causes a dramatic reprogramming in cellular metabolism. Heat shock affects the cells at the level of the nucleic acids, the membrane, and the cytoskeleton. Heat shock induces a significant reduction in normal transcription and translation processes. The next event in the heat shock response pathway is the activation of heat shock transcription factor (HSF). Activated HSF binds to the heat shock element and induces the synthesis of Hsps (1–4). The production of Hsps induces a transient thermotolerance.

Heat shock has been shown previously to alter the phosphorylation of some cellular proteins in several different systems, including soybean seedlings (5) and mammalian cell lines (6). Heat shock elevates the level of protein phosphorylation in several cell lines (7). Recently, there has been increasing evidence that cellular stress responses are regulated by protein kinases. Heat shock activates a number of protein kinases, including p38/HOG1 kinase (8), Jun kinase (9), MAPK (10–12), ribosomal S6 kinase (13), phosphatidylinositol 3-kinase, c-Src tyrosine kinase (14), MAP kinase activated protein kinase 1 (MAPKAP kinase 1) (15), and MAPKAP kinase 2 (16). However, only a few of the substrates of these kinases have been identified. For example, RNA polymerase II (17, 18), histone H1 (19), Hsp (20), eukaryotic translation initiation factor 2 (21), and HSF (22–24) have been identified as substrates phosphorylated by heat shock.

In this study, we examined the global phosphorylation changes after heat shock in a radiation-induced fibrosarcoma cell line, RIF-1, and its therootolerant derivative, TR-RIF-1. Identifications of phosphorylated proteins induced by heat shock were performed by proteomics combined with two-dimensional gel electrophoresis, Western analysis using anti-phospho-

§ The abbreviations used are: Hsp, heat shock protein; HSF, heat shock factor; PTK, protein-tyrosine kinase; RIF, radiation-induced fibrosarcoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFR, epidermal growth factor receptor; MALDI-TOF MS, matrix-assisted laser desorption/ionization mass spectrometry; MAP, mitogen-activated protein; MAPK, MAP kinase; hnRNP, heterogeneous nuclear ribonucleoprotein. Throughout this manuscript, the term “protein phosphorylation” is used in short for “protein tyrosine phosphorylation” and “phosphorylation” for “tyrosine phosphorylation.”
glycerol, 150 mM KCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride at 14,500 rpm. The supernatant (nucleus fraction) was added to the pellet, which was resuspended in half-packed cell volume of low salt buffer (20 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM/mg aprotinin, 10 g/ml pepstatin A, 20 g/ml/μm leupeptin, 5 g/ml NaNO3, 5 mM NaF) to a final volume of 3 times the original packed cell volume in ice. After centrifugation, the supernatant (cytosol fraction) was separated, and the pellets were stored at -80°C until use.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Heat Treatment—**Radiation-induced fibrosarcoma RIF-1 (25) and thermotolerant TR-RIF-1 cell lines derived from RIF-1 (gifts from Dr. G. M. Hahn) were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin G at 37°C in an atmosphere of 5% CO2, 95% air. For the heat treatment studies, monolayers of cells grown in tissue culture dishes were incubated at 45 ± 0.1°C in a water bath.

**Profiling of Protein Synthesis by [35S]Methionine Pulse Labeling—**Patterns of cellular protein synthesis after heat shock were examined by pulse labeling with [35S]methionine in methionine-free RPMI 1640 media for 1 h. The labeled proteins were separated on SDS-PAGE gels, autoradiographed, and quantified by BAS2500 (Fuji photo film).

**Immunoprecipitation—**The cells were treated with or without heat shock at 45°C for 30 min and subsequently lysed in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM/mg aprotinin, 10 g/ml pepstatin A, 20 g/ml/μm leupeptin, 5 g/ml NaNO3, 5 mM NaF) to a final volume of 3 times the original packed cell volume in ice. After centrifugation, the supernatant (cytosol fraction) was separated, and the pellets were resuspended in half-packed cell volume of low salt buffer (20 mM HEPES, pH 7.4, 15% glycerol, 2% SDS, 10 mg/ml dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml/μg leupeptin, 5 μM NaNO3, 5 mM NaF). High salt buffer (same as low salt buffer except that 0.02 M KCl replaced with 1.2 M KCl) was added to one-half of the packed cells in volume in dropwise, incubated in ice for 30 min, and centrifuged for 30 min at 14,500 rpm. The supernatant (nucleus fraction) was added to the cytosol fraction. The protein concentrations were measured by the Bradford assay. Equal amounts of proteins (700 μg) were added to an immunoprecipitation buffer containing 20 mM HEPES, pH 7.4, 15% glycerol, 150 mM KCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml/μg leupeptin, 5 μM NaNO3, 5 mM NaF, 5 μl of anti-phosphotyrosine antibody was added and incubated at 4°C for 3 h. 20 μl of protein A/G beads were then added and incubated for 1 h. The precipitated immune complexes were washed three times with the immunoprecipitation buffer. Proteins from control and heat-shocked cells were separated by SDS-PAGE under reducing conditions, transferred to NC membrane, and probed with polyclonal antibody of GAPDH (gift from Dr. K. S. Kwon) and monoclonal antibody to HSC70/HSP70 (StressGen). The immune complexes were detected with Amersham Biosciences ECL kit and LAS-1000S (Fuji photo film).

**Two-dimensional Gel Electrophoresis and Immunoblot Analysis—**The samples were mixed for 30 min at room temperature with a buffer containing 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 10 μg/ml/μg leupeptin, 5 μM NaNO3, 5 mM NaF, 5 μl of anti-phosphotyrosine antibody and washed at 4°C for 3 h. 20 μl of protein A/G beads were then added and incubated for 1 h. The precipitated immune complexes were washed three times with the immunoprecipitation buffer. Proteins from control and heat-shocked cells were separated by SDS-PAGE under reducing conditions, transferred to NC membrane, and probed with polyclonal antibody of GAPDH (gift from Dr. K. S. Kwon) and monoclonal antibody to HSC70/HSP70 (StressGen). The immune complexes were detected with Amersham Biosciences ECL kit and LAS-1000S (Fuji photo film).

**In-gel Digestion and Mass Spectrometric Analysis—**The cellular proteins were separated on two-dimensional gel electrophoresis and stained with Coomassie Blue or Silver. Each spot was in-gel digested with trypsin and subjected to mass spectrometry analysis. The extracted peptides were analyzed by a PerSeptive Biosystems Voyager DE PRO mass spectrometer in the linear positive-ion mode. The MALDI spectra were obtained from a 10-kDa insulin standard and used as a standard.
Peptide mixtures were analyzed with MALDI-TOF MS using a delayed ion extraction and ion mirror reflector mass spectrometer (Voyager-DE STR; Applied Biosystems, Inc.). External calibration was carried out using Sequazyme Peptide Mass Standard Kit (Perspective Biosystems) and internal calibration, by using the autolytic peaks of trypsin. This procedure typically results in mass accuracies of 50 ppm. For inter-
pretation of the mass spectra, we used the MS-Fit program available on the web site of the University of California, San Francisco (prospector.ucsf.edu/).

Prediction of Phosphotyrosine Motifs—The phosphorylation sites were predicted by NetPhos program from the website (www.cbs.dtu.dk/services/NetPhos) having low stringency and ScanProsite (www.expasy.ch/tools/scnpsite.html) having high stringency. PTKs involved in phosphorylations, and the possible binding proteins of tyrosine phosphorylated proteins were predicted by Scansite program at the website (scansite.mit.edu/).

RESULTS

Heat Shock-induced Protein Phosphorylations—To determine whether protein phosphorylations might be involved in heat shock response and thermotolerance, we first examined the induction of tyrosine phosphorylation in control RIF-1 cells and their thermotolerant derivatives, TR-RIF-1 cells. Both RIF-1 and TR-RIF-1 cells were exposed to heat shock at 45 °C for 15 or 45 min. After each treatment, tyrosine-phospho-
Protein synthesis was monitored by [35S]methionine pulse labeling in RIF-1 cells at 45 °C for 45 min, and then labeled with [35S]methionine for 1 h to determine the kinetics of protein synthesis. Some of the phosphorylated proteins were common to the two cell lines and showed a dose-dependent manner in both RIF-1 and TR-RIF-1 cells.

Next we carried out metabolic labeling of the newly synthesized proteins to determine the kinetics of protein synthesis after exposure to heat shock at 45 °C for 30 min, then labeled with [35S]methionine for 1 h. The film images were scanned using ImageMaster LabScan version 3.00 software connected to Umax scanner. The identified proteins were listed and grouped by their known functions in Table I and as follows: 19 proteins (23 spots) functioning in amino acid biosynthesis, 5 proteins (6 spots) acting in protein folding, 13 proteins (17 spots) functioning in transcription and translation, 4 proteins acting in amino acid biosynthesis, 2 proteins relating to oxidation and reduction, 2 channel proteins, 8 (10 spots) signaling molecules, 11 proteins (16 spots) functioning in cell motility or structure, 13 protein (18 spots) acting in energy metabolism, and 6 miscellaneous proteins. Of these, known tyrosine-phosphorylated proteins are as follows: hnRNP A2/B1 and GAPDH were phosphorylated by c-Src (29) and EGFRK (30), respectively. Annexin II cloned as PTK substrate has been identified as phosphorylated proteins both in RIF-1 and TR-RIF-1 cells: 52 spots in pI range of 4–7 and 41 spots in pI range of 5–6. Immunostained spots detected in any one of the immunoblot analyses were overlaid and are depicted by numbered arrows in the silver-stained gel (Fig. 2A) and Coomassie Blue-stained gel (Fig. 2C). The corresponding immunostained gels were shown in Fig. 2B and D. Examples of heat shock-induced tyrosine phosphorylation changes detected with immunostaining are shown in Fig. 2E.

Protein spots detected on the immunoblot were cut out from the corresponding gel, subjected to in-gel digestion with trypsin, and mass peptide fingerprint analyses were conducted. Low abundance proteins (weakly stained with silver) were identified by pooling spots from more than three gels. The obtained mass data were fitted by MS-Fit database search analysis. This allowed us to identify 81 of 93 proteins (64 new phosphorylated proteins and 20 phosphorylated proteins that were previously reported). The identified proteins were listed and grouped by their known functions in Table I and as follows: 5 proteins (6 spots) acting in protein folding, 13 proteins (17 spots) functioning in transcription and translation, 4 proteins acting in amino acid biosynthesis, 2 proteins relating to oxidation and reduction, 2 channel proteins, 8 (10 spots) signaling molecules, 11 proteins (16 spots) functioning in cell motility or structure, 13 protein (18 spots) acting in energy metabolism, and 6 miscellaneous proteins. Of these, known tyrosine-phosphorylated proteins are as follows: hnRNP A2/B1 and GAPDH are phosphorylated by c-Src (29) and EGFRK (30), respectively. Annexin II cloned as PTK substrate has been identified as

| Spot No. | Identified protein | NCBI Accession No. | Mass | pI | Coverage |
|----------|-------------------|--------------------|------|----|----------|
| 16       | ATP synthase 60S subunit, ATPase 2 | 282222             | 56,380.1 | 5.14 | 38       |
| 19       | Proteasome 28S subunit, ATPase 2 | 13529470           | 48,648.3 | 5.72 | 38       |
| 28       | Galactokinase     | 8650476             | 42,295.6 | 5.17 | 23       |
| 53       | Similar to mitochondrial aconitase | 13345538           | 85,464.2 | 8.08 | 29       |
| 58       | Pyruvate kinase, M2 isozyme | 2506976            | 57,887.3 | 7.17 | 35       |
| 63       | Pyrophosphate kinase | 202423             | 44,536.8 | 7.54 | 28       |
| 64       | Phosphoglycerate kinase | 202423             | 44,536.8 | 7.54 | 28       |
| 85       | ErbB3-related receptor tyrosine kinase | 6671539 | 39,356.1 | 8.30 | 29       |
| 70       | GAPDH             | 6679937             | 35,810.2 | 8.43 | 27       |
| 71       | GAPDH             | 6679937             | 35,810.2 | 8.43 | 27       |
| 76       | Lactate dehydrogenase A | 538138             | 36,498.9 | 7.61 | 36       |
| 77       | Creatine kinase, M chain | 125306             | 43,945.3 | 6.58 | 15       |
| 84       | Pyruvate kinase isozyme M2 | 1363219            | 57,861.4 | 7.58 | 26       |
| 85       | Pyruvate kinase isozyme M2 | 1363219            | 57,861.4 | 7.58 | 26       |
| 87       | Aldolase reductase | 3046247             | 35,718.5 | 6.71 | 20       |
| 91       | Similar to phosphoglycerate mutase 1 | 12805529 | 28,832.2 | 6.68 | 39       |
| 92       | Triosephosphate isomerase | 54855              | 26,659.5 | 6.90 | 40       |
| 93       | Triosephosphate isomerase | 54855              | 26,659.5 | 6.90 | 34       |

**Miscellaneous**

| Spot No. | Identified protein | NCBI Accession No. | Mass | pI | Coverage |
|----------|-------------------|--------------------|------|----|----------|
| 18       | Putative          | 12850298           | 52,769.2 | 5.75 | 21       |
| 27       | Reticulocalbin    | 220582             | 38,113.4 | 5.19 | 24       |
| 30       | RIKEN cDNA 2410174K12 gene | 14318755 | 38,113.4 | 5.19 | 24       |
| 41       | Putative          | 12848170           | 38,937.5 | 6.41 | 36       |
| 52       | β-Proteasome subunit | 1762977            | 29,116.4 | 5.47 | 34       |
| 60       | RIKEN cDNA 2700043D08 gene | 13435984 | 55,718.5 | 6.71 | 20       |

10, 13, 14, 23, 25, 31, 34, 42, 44, 45, 57, 89*  
* These spot numbers are unidentified.
Fig. 3. Quantification of protein tyrosine phosphorylation. A, comparison of basal phosphorylations in control RIF-1 cells and thermotolerant TR-RIF-1 cells. Immunostained spots with phosphotyrosine antibody in unstressed RIF-1 (black bars) and TR-RIF-1 (white bars) cells were quantified, and their intensities were normalized to the intensities of RIF-1 cells and expressed as a relative intensity. B and C, kinetics of the phosphorylation in tyrosine residues in various spots during recovery after heat shock at 45 °C for 30 min. Immunostained spots of heat shock-stressed RIF-1 (B) and TR-RIF-1 (C) were quantified, and their intensities were normalized to control without heat shock and expressed in relative magnitude as a relative intensity. White bars, control cells; black bars, cells immediately after heat shock; hatched bars, 4 h recovered cells after heat shock; grey bars, 24 h recovered cells. Bars more than 10-fold were abbreviated.
major substrates for retrovirus encoded PTK pp60 v-Src (31), platelet-derived growth factor (32), insulin (33), and hepatocyte growth factor/scatter factor (34) receptor tyrosine kinases. Hsp70 was revealed as phosphorylation at Tyr-524 in COS-1 cells that corresponded with Tyr-525 in mouse Hsp70 (35). Aldolase 1A and lactate dehydrogenase A were shown that phosphorylated in Tyr-361 (Tyr-364 in mouse aldolase 1A) (36) and Tyr-238 (Tyr-239 in mouse lactate dehydrogenase A) (37), respectively.

A few proteins could not be identified because these were low abundant proteins (<100 fmol) and poorly detected with silver staining, or because peptide mass fingerprints with sound spec-
TABLE II
Comparison of protein tyrosine phosphorylation after heat shock in RIF-1 and TR-RIF-1 cells

| Spot No. | Identified protein | TR/RIF | RIF-1 | TR-RIF-1 |
|----------|-------------------|--------|-------|---------|
|          |                   | c Imm  | 4h    | 24h     |
|          |                   | c Imm  | 4h    | 24h     |
| PROTEIN FAMILY 1 | | | |
| Proteins that act as chaperones/mediators of protein folding | | | |
| 1 Heat shock protein 70 cognate | +++ | 1 1 1 1 | 1 0 1 0 |
| 2 Heat shock protein 70 cognate | + + | 1 ++ ++ 0 | 1 1 ++ 1 |
| 3 HSP70 | + | 1 1 1 ++++ | 1 1 ++ + |
| 5 CCT (chaperonin-containing TCP-1)e-subunit | + | 1 1 + 1 | 1 ++ 0 |
| Proteins that function in transcription and translation | | | |
| 56 L-protein (H. sapiens) | +++ | 1 +++ +++ +++ | 1 + 0 + |
| Proteins that are related to oxidation or reduction | | | |
| 51 Nonessential glutathione peroxidase | ++ | 1 + ++ ++ | 1 1 1 1 |
| 90 Glutathione S-transferase homolog | +++ | 1 0 ++ 0 | 1 ++ ++ + |
| Proteins that compose channels | | | |
| 85 Voltage-dependent anion channel 2 | ++ | 1 0 ++ + | 1 + + 1 |
| Proteins that act as signaling molecules | | | |
| 32 CPP32 | + | 1 1 1 ++ | 1 + 1 0 |
| 35 TGF-β receptor-binding protein | ++ | 1 + ++ + | 1 1 ++ 1 |
| 50 Prohibitin, B-cell receptor-associated protein (BAP) 32 | +++ | 1 ++ +++ + | 1 0 1 1 |
| Proteins that function in cell motility or structure | | | |
| 46 Tropomyosin 5 | + | 1 + ++ ++ | 1 + + 1 |
| 49 Capping protein β-subunit, isoform 2 | +++ | 1 ++ +++ ++ | 1 1 + 1 |
| Proteins that act in energy metabolism | | | |
| 58 Pyruvate kinase, M2 isozyme | ++ | 1 + +++ ++ | 1 + ++ ++ |
| 70 GAPDH | ++ | 1 + 0 1 | 1 1 1 1 |
| 84 Pyruvate kinase isozyme M2 | +++ | 1 1 +++ ++ | 1 + 0 + |
| 85 Pyruvate kinase isozyme M2 | +++ | 1 1 ++ + | 1 + 0 0 |
| 92 Triosephosphate isomerase | +++ | 1 0 ++++++ + | 1 + + 1 |
| Miscellaneous | | | |
| 30 RIKEN cDNA 2410174K12 gene | ++ | 1 + +++ ++ | 1 1 1 0 |
| 41 Putative | ++ | 1 + ++ +++ | 1 + + 1 |
| 42 Unidentified | +++ | 1 + +++ ++ | 1 1 0 0 |
| 44 Unidentified | ++++ | 1 1 ++ ++++ | 1 0 0 0 |
| 45 Unidentified | ++ | 1 1 ++ + | 1 0 0 0 |
| PROTEIN FAMILY 2 | | | |
| Proteins that function in transcription and translation | | | |
| 72 hnRNP A2/B1 | – | 1 + + + | 1 + +++ +++ |
| 75 TIS | – | 1 1 1 1 | 1 ++ ++ + |
| 78 TIS | – | 1 0 0 1 | 1 +++ + + |
| Proteins that act in amino acid biosynthesis | | | |
| 61 Glutamate dehydrogenase | – | 1 + 0 0 | 1 ++ 1 +++ |
| 66 Pyruvate dehydrogenase | – | 1 0 + + | 1 ++ ++ + |
| Proteins that compose channels | | | |
| 80 Voltage-dependent anion channel 1 | – | 1 1 0 + | 1 + + + |
| Proteins that act as signaling molecules | | | |
| 24 Annexin A7 | – | 1 0 1 + | 1 + + + |
| 67 MAP kinase kinase | – | 1 1 0 1 | 1 ++ ++ + |
| 68 MAP kinase kinase | – | 1 1 1 1 | 1 ++ ++ + |
| 74 Annexin II, lipocortin II | – | 1 + 1 + | 1 ++ ++ + |
| 82 L-34 protein (AA 1–264) | – | 1 1 0 1 | 1 1 + + |
| Proteins that function in cell motility or structure | | | |
| 15 β-Tubulin | – | 1 0 0 1 | 1 + + + |
| 21 Vimentin | – | 1 1 1 1 | 1 + + + |

Comparison of protein tyrosine phosphorylation after heat shock in RIF-1 and TR-RIF-1 cells divided by the level of control RIF-1 cells in normal state; c, control; Imm, immediately after heat shock; 4h, 4 h recovery after heat shock; 24h, 24 h recovery after heat shock; 1, relative tyrosine phosphorylation level of control cells; 0, decreased intensity; +, 2–5-fold increase; ++, 5–10-fold increase; ++++, 10–100-fold increase: +++, more than 100-fold increase: –, 2–5-fold decrease; –, 5–10-fold decrease; ––, 10–100 fold decrease; ––, less than 2-fold difference.

Proteins that function in cell motility or structure

Heat Shock Changes in the Levels of Protein Phosphorylation—To determine the possible molecules involved in heat shock response and thermotolerance, we examined the kinetics of protein tyrosine phosphorylations in both RIF-1 and thermotolerant TR-RIF-1 cells during recovery after heat shock. Cellular proteins obtained from cells exposed to heat shock at 45 °C for 30 min and then recovered for 0, 4, and 24 h were separated on two-dimensional gel and detected with immunoblotting with phosphotyrosine antibody. Each spot was
Proteins that act in energy metabolism

- ATP synthase β-subunit
- GAPDH
- Lactate dehydrogenase A
- Aldolase reductase
- Similar to phosphoglycerate mutase

Miscellaneous

- Unidentified
- Unidentified

PROTEIN FAMILY 3

Proteins that act as chaperones/mediators of protein folding

- Similar to ER-60 protease

Proteins that function in transcription and translation

- Heat shock transcription factor 2
- hnRNP H
- Murin homolog of human rtp-3 (hnRNP H)
- Ribonucleoprotein F
- Nucleolar protein
- Eukaryotic translation elongation factor1-δ
- Eukaryotic translation elongation factor1 β2
- Similar to replication protein A2
- L-protein (H. sapiens)
- hnRNP A2/B1
- hnRNP A2/B1
- NonO

Proteins that act in amino acid biosynthesis

- Ornithine-α-oxidation transferase
- Inosine-5-monophosphate dehydrogenase 2

Proteins that act as signaling molecules

- MAP kinase kinase
- G protein β-subunit-like

Proteins that function in cell motility or structure

- Vimentin
- Vimentin
- Vimentin
- BAF65a
- γ-Actin
- β-Tropomyosin
- Capping protein α-1 subunit
- α-Tropomyosin

Proteins that act in energy metabolism

- Proteasome 26 S subunit, ATPase 2
- Galactokinase
- Phosphoglycerate kinase
- Phosphoglycerate kinase
- Aldolase 1, A isoform
- Creatine kinase, M chain
- Triosephosphate isomerase

Miscellaneous

- Unidentified
- Unidentified
- Putative
- Unidentified
- β-Proteasome subunit
- RIKEN cDNA 2700043D08 gene

Because phosphorylations may modulate protein activity, the degree of protein phosphorylation is probably a better marker of cellular status than protein levels. To determine the molecules involved in heat shock signaling and thermotoler-

semi-quantified using ImageMaster two-dimensional version 3.01 software. To compensate the false positive or negative caused by the different efficiency of each gel in transferring proteins from gel to membrane, blocking of nonspecific binding, probing with antibody, exposure time of chemiluminescence, and so on, we loaded same amount of phosphotyrosine molecular weight marker in each gel. We converted the measured intensity of each spot into fold number over the intensity of standard marker. We calculated the relative intensity of each sample to compare the intensity with control sample without heat shock and represented in Fig. 3.

Because phosphorylations may modulate protein activity, the degree of protein phosphorylation is probably a better marker of cellular status than protein levels. To determine the molecules involved in heat shock signaling and thermotoler-

![Heat shock-induced tyrosine phosphorylation of GAPDH and heat shock protein 70 cognate.](image)
Table III
A list of predicted tyrosine phosphorylation sites using NetPhos and ScanProsite prediction programs
The table lists proteins identified in this study and predicted phosphotyrosine residues using NetPhos (www.cbs.dtu.dk/services/NetPhos/) and ScanProsite (www.expasy.ch/tools/scanpsite.html). The table also shows previously reported tyrosine phosphorylated proteins by references.

| Spot No. | Identified protein | NetPhos prediction site | ScanProsite prediction site | Ref. |
|----------|-------------------|-------------------------|----------------------------|------|
| 1        | Heat shock protein 70 cognate | 15, 41, 288, 431, 525, 545 | 525 | 35 |
| 2        | Heat shock protein 70 cognate | 15, 41, 288, 431, 525, 545 | 525 | 35 |
| 3        | HSP70 | 15, 41, 431, 424 | 525 | 51 |
| 5        | CCT (chaperonin-containing TCP-1) e-subunit | 12, 137, 274 | 503 | 52 |
| 6        | Similar to ER-60 protease | 67, 95, 115, 269, 416, 445, 454 | 115, 416, 479 | 52 |
| 62       | 47-kDa heat shock protein | 153, 346, 370, 382 | 382 | 382 |
| 4        | Heat shock transcription factor 2 | 279, 293, 362 | 249 | 52 |
| 11       | hnRNP H | 210, 243, 253, 266, 276, 306, 372, 376, 433 | 266, 306 | 52 |
| 12       | Murin homolog of human ftp-3 (hnRNP H) | 210, 243, 249, 253, 366, 376, 306, 372, 376, 433, 444 | 266, 306 | 52 |
| 20       | ribonucleoprotein F | 11, 194, 210, 253, 266, 306, 372, 414 | 306 | 52 |
| 36       | Nucleolar protein | 18, 26 | 18 | 52 |
| 37       | Eukaryotic translation elongation factor 1-5 | 24, 28, 213 | 213 | 52 |
| 48       | Similar to replication protein A2 | 14, 20, 125, 256 | 125, 256 | 52 |
| 55       | L-protein (Homo sapiens) | 61, 132, 173, 181, 254, 293, 297, 302, 332, 344, 399 | 254 | 52 |
| 56       | L-protein (Homo sapiens) | 61, 132, 173, 181, 254, 293, 297, 302, 332, 344, 399 | 254 | 52 |
| 72       | hnRNP A2/B1 | 123, 222, 232, 264, 275, 288 | 288 | 52 |
| 73       | hnRNP A2/B1 | 123, 222, 232, 264, 275, 288 | 288 | 52 |
| 75       | TIS | 128, 244, 253, 262 | 262 | 52 |
| 78       | TIS | 128, 244, 260, 289, 314 | 314 | 52 |
| 79       | hnRNP A2/B1 | 123, 222, 232, 264, 275, 288 | 288 | 52 |
| 80       | NonO | 160, 269 | 269 | 52 |
| 86       | RNA-binding protein a-CP1 | 207 | 207 | 52 |
| 22       | Ornithine-ox-o-acid aminotransferase | 45, 50, 126, 158, 194, 209, 245, 299 | 299 | 52 |
| 59       | Inosine-5-monophosphate dehydrogenase 2 | 4, 41, 258, 294, 430 | 430 | 52 |
| 61       | Glutamate dehydrogenase | 193, 240, 288, 429, 539 | 429, 539 | 52 |
| 66       | Pyrolysosomal aspartate aminotransferase | 316, 401, 420 | 401, 420 | 52 |
| 51       | Nonessential glutathione peroxidase | 217 | 217 | 52 |
| 50       | Glutathione S-transferase homolog | 97, 108, 160, 174 | 160, 174 | 52 |
| 51       | Proteins that are related to oxidation or reduction | 45, 50, 126, 158, 194, 209, 245, 299 | 299 | 52 |
| 50       | Proteins that compose channels | 45, 50, 126, 158, 194, 209, 245, 299 | 299 | 52 |
| 80       | Voltage-dependent anion channel 1 | 7, 62, 118 | 118 | 52 |
| 88       | Voltage-dependent anion channel 2 | 19, 74 | 74 | 52 |
| 24       | Annexin A7 | 6, 358, 377, 401, 402 | 402 | 52 |
| 32       | CPP32 | 32, 41, 126, 147, 195, 274 | 195, 274 | 52 |
| 35       | TGF-β receptor-binding protein (BAP) 32 | 139, 300, 308, 318 | 308, 318 | 52 |
| 67       | MAP kinase kinase | 339 | 339 | 52 |
| 68       | MAP kinase kinase | 339 | 339 | 52 |
| 69       | MAP kinase kinase | 339 | 339 | 52 |
| 74       | Annexin II, lipocortin II | 24, 30, 151, 188, 275, 327 | 327 | 52 |
| 81       | G protein β-subunit-like | 52 | 52 | 52 |
| 82       | L-34 protein (AA 1–264) | 52 | 52 | 52 |

Table II summarizes these studies. We sorted the proteins into three groups depending on the ratio of phosphorylation degree of basal level without heat shock in TR-RIF-1 to in RIF-1 (TR/RIF in Table II).

The first group of proteins (Table II, PROTEIN FAMILY 1) and the second group of proteins (Table II, PROTEIN FAMILY 2) show higher basal tyrosine phosphorylation levels in TR-RIF-1 cells than in control RIF-1 cells. These proteins responded sensitively to heat shock and significantly phosphorylated only in RIF-1 cells. This group of proteins showed increased extents of tyrosine phosphorylations in TR-RIF-1 cells and only phosphorylated by heat shock in RIF-1 cells. It is possible that the phosphorylated forms of the proteins are involved in maintaining cellular thermotolerance. These include 4 chaperones/mediators of protein folding, 2 oxido-reduction related proteins, 2 transcription and translation related proteins, 3 signaling molecules, 1 protein that serves as an ion channel, 2 proteins influencing cell motility and structure, 5 proteins playing roles in energy metabolism, and 5 miscellaneous or unidentified.

The third group of proteins showed higher basal tyrosine phosphorylation levels in RIF-1 cells than in TR-RIF-1 cells (Table II, PROTEIN FAMILY 3). These proteins responded sensitively to heat shock only in TR-RIF-1 cells and were dramatically phosphorylated by heat shock. The dephosphorylated forms of the proteins may make the cells less sensitive to heat shock. Heat shock-induced tyrosine phosphorylation of the pro-
The third group of proteins showed similar basal tyrosine phosphorylation levels between RIF-1 and TR-RIF-1 cells, and they similarly responded to heat shock and similar increase of tyrosine phosphorylation in both cells (Table II, PROTEIN FAMILY 3). This group of proteins is phosphorylated by heat shock in both RIF-1 and TR-RIF-1 cells, although minor kinetic differences exist. These proteins may directly respond to heat shock and may be involved in heat shock signaling pathways regardless of thermotolerance. The group of proteins includes the following: 1 that acts as chaperone/mediator, 12 proteins functioning in transcription and translation, 2 acting in amino acid biosynthesis, 2 acting in signaling, 8 cytoskeletal proteins, 7 acting in energy metabolism, and 2 unidentified proteins.

Before the quantification, we expected that most of the proteins would show increased levels of protein tyrosine phosphorylation (Fig. 1A). However, some proteins such as the 47-kDa heat shock protein, α-tubulin isotype M-α-6, lamin A, and an unidentified protein showed decreased levels of tyrosine phosphorylation as a function of time after heat shock. This suggests that some kinases may be inactivated by heat shock or some phosphatases may be activated by heat shock. It would be of interest to identify these kinase and phosphatases involved in heat shock signaling pathway.

To determine the reliability of our semi-quantitative analysis, we performed immunoprecipitation analysis with anti-phosphotyrosine antibody in heat-shocked RIF-1 and TR-RIF-1 cells. Heat shock induced dramatic phosphorylation on GAPDH in heat-shocked TR-RIF-1 cells (Fig. 4). Although the phosphorylation levels of control cells were detectable in two-dimensional gel analysis, it is hard to detect in Fig. 4. It may be due to a small fraction of phosphorylation on GAPDH in control RIF-1 and TR-RIF-1 cells or a small amount of anti-phosphotyrosine antibody used in the immunoprecipitation. In the case of HSC70, heat shock increased tyrosine phosphorylation in both cell lines, although the total amount of phosphorylated protein is higher in TR-RIF-1 cells. These results were coincident with the quantitative analysis results performed as presented in Fig. 3. In Fig. 3B and C, spot 70 (GAPDH) did not show prominent changes in both RIF-1 and TR-RIF-1 cells and spot 71 (GAPDH) of TR-RIF-1 cells showed an increased level of phosphorylation intensity after a 4-h recovery more than 5-fold compared with RIF-1 cells. Heat shock increased the phosphorylation level of spot 2 (HSC70) in both RIF-1 and TR-RIF-1 cells after a 4-h recovery. The basal levels of tyrosine phosphorylation of GAPDH and HSC70 in Fig. 4 were well matched with the quantitative results in Fig. 3A. In addition,
we detected tyrosine phosphorylation of hnRNP A2/B1 in RIF-1 cells and vimentin in Rat2 cells by immunoprecipitation and Western blot analysis (data not shown). Although we did not examine all of the listed proteins, the specificity of anti-phosphotyrosine antibody used in Western blot analysis and accuracy of computational quantification are quite reliable.

**Prediction of Possible Tyrosine Phosphorylation Sites of Each Protein**—Large scale analysis of 64 phosphorylated proteins by

### TABLE IV

A list of predicted tyrosine phosphorylation sites using Scansite prediction program

The table lists proteins identified in this study and predicted phosphotyrosine residues using Scansite (scansite.mit.edu/). The table also shows the possible protein tyrosine kinases that phosphorylate the proteins and the possible proteins that bind to the tyrosine-phosphorylated proteins.

| Protein Description                                      | PDZ  | SH2/PTB |
|-----------------------------------------------------------|------|---------|
| heat shock protein 70 cognate                            | 67   | 314     |
| heat shock protein 70 cognate                            | 67   | 314     |
| HSP70                                                    | 41   | 149     |
| heat shock transcription factor 2                        | 293  | 293     |
| CCT (chaperonin containing TCP-1 epsilon subunit)        | 454  | 293     |
| similar to ER-60 protease                                | 115  | 264     |
| vimentin                                                 | 115  | 264     |
| vimentin                                                 | 115  | 264     |
| unidentified                                             | 115  | 264     |
| hnRNP H                                                  | 306  | 306     |
| muin homolog of human fip-3 (hnRNP H)                    | 376  | 376     |
| unidentified                                             | 376  | 376     |
| unidentified                                             | 376  | 376     |
| beta-tubulin                                             | 42   | 209     |
| A TP synthase beta-subunit                               | 243  |         |
| BAF53a                                                   | 243  |         |
| putative                                                 | 243  |         |
| proteasome 20s subunit, ATPase 2                         | 192  |         |
| hnRNP F (rat)                                            | 306  | 306     |
| vimentin                                                 | 246  | 243     |
| ornithine-oxo-aicin aminotransferase                     | 209  |         |
| unidentified                                             | 209  |         |
| unidentified                                             | 209  |         |
| gamma actin                                              | 233  |         |
| reilucalin                                                | 233  |         |
| galactokinase                                            | 233  |         |
| beta-tropomysin                                          | 162  | 162     |
| RIKEN zDNA 2410174K12 gene                               | 336  | 336     |
| unidentified                                             | 336  | 336     |
| CTP32                                                    | 336  | 336     |
| capping protein alpha 1 subunit                          | 336  | 336     |
| TGF-beta receptor binding protein                        | 158  | 158     |
| nuclear protein                                           | 158  | 158     |
| eukaryotic translation elongation factor 1-delta          | 26   | 126     |
| alpha tubulin isotype M-alpha-6                          | 449  | 449     |
| alpha tropomysin                                         | 162  | 162     |
| alpha tropomysin                                         | 162  | 162     |
| putative                                                 | 162  | 162     |
| putative                                                 | 162  | 162     |
| tropomysin 5                                             | 126  | 126     |
| eukaryotic translation elongation factor 1 beta 2         | 126  | 126     |
| similar to replication protein A2                        | 126  | 126     |
| capping protein beta subunit, isoform 2                   | 79   |         |
| prohibitin or B-cell associated protein (BAP)32           | 114  |         |
| nonseleum glutathione peroxidase                         | 114  |         |
| beta proteasome subunit                                   | 114  |         |
| similar to mitochondrial aconitase                       | 544  | 544     |
| lamin A                                                  | 481  |         |
| L-protein (homo sapiens)                                 | 357  | 327     |
| L-protein (homo sapiens)                                 | 357  | 327     |
| unidentified                                             | 357  | 327     |
| pyruvate kinase, M2 isozyme                              | 544  | 544     |
| pyruvate kinase, M2 isozyme                              | 544  | 544     |
| glutamate dehydrogenase                                  | 269  |         |
| 40K heat shock protein                                   | 370  |         |
heat shock were performed by computer-assisted program. This study allowed us to predict the possible phosphorylation sites, possible protein kinases involved, and the possible proteins that bind to phosphorylated proteins. Prediction of phosphorylation sites were performed by two computer programs including NetPhos (www.cbs.dtu.dk/services/NetPhos/) and ScanProsite (www.expasy.ch/tools/scnpsite.html) shown in Table III. The discrepancies between the two programs arise from the different algorithms. ScanProsite predicts the phosphorylation sites based on the limited motifs (RK)(X2)(DE)(X3)Y or (RK)(X3)(DE)(X2)Y (where Y is the phosphorylation site), whereas NetPhos is based on neural network method which tends to predict the false positive sites. Table IV lists the predicted phosphotyrosine residues with possible phosphotyrosine kinase or phosphotyrosine recognition motifs using Scansite (scansite.mit.edu/). The predicted site was characteristic of each of the program. Steen et al. (38) reported that such predictions should be done very cautiously. All three programs predicted the same phosphorylation site in only one protein, Tyr-306 in hnRNP H (spots 11 and 12). However, as the programs use unique algorithms for prediction of phosphorylation sites, combining and comparing the results from the three programs should give useful information. In fact, the programs can predict the tyrosine phosphorylation sites as reported experimentally; HSC70 (spots 1 and 2), aldolase A isofrom (spot 65), and lactate dehydrogenase A (spot 76) were previously known as tyrosine-phosphorylated proteins and Tyr-525 (Tyr-524 in COS-1 cells) (35), Tyr-364 (Tyr-361 in rabbit liver cell) (36), and Tyr-239 (Tyr-238 in Rous sarcoma virus-transformed cell) (37) were the tyrosine phosphorylation sites, respectively. At least two of the three programs predicted the known sites correctly.

Scansite predicted not only tyrosine phosphorylation sites but also tyrosine kinase that phosphorylated the predicted sites and binding proteins of phosphorylated motifs. From the results, we can postulate the heat shock-activated signaling pathways. The numbers presented at the bottom of Table IV suggest that the sum of the numbers of proteins that are predicted as sites for each of kinase or phosphotyrosine recognition motif. It appears that the bigger the number, the higher the possibility that the kinases and phosphotyrosine recognition motif-containing proteins were activated. Except for PDZ class 2 phosphotyrosine recognition motif, all kinases and phosphotyrosine recognition motifs were predicted from 3 to 13. It means the listed protein tyrosine kinases and proteins that recognize phosphotyrosine motifs can be activated in response to heat shock. Activation of EGFR and c-Src (14) and induction of epidermal growth factor (40) and basic fibroblast growth factor (41) by heat shock were reported previously. p56 Lck was activated in lymphocyte in response to oxidants, heavy metals, and heat shock (42). As Lck and Nck are immune cell-specific kinases, homologs of Lck and Nck may act in RIF-1 and TR-RIF-1 cells in response to heat shock. Also, heat shock induced activation of Ras-Raf-MAPK and Shc-Grb2 pathways (43). In this study, 13 sites and 6 sites were predicted as Grb2-SH2 and Shc-SH2 recognition sites, respectively. The most frequently predicted site was PLCγ/SH2 binding domain. Heat shock-induced activation of PLC was briefly reported previously (44–47).

**DISCUSSION**

These studies have identified a number of proteins involved in heat shock phenomena by proteome analysis. We focused on phosphorylation based on the previous reports (7) that heat shock activates various kinases and induces tyrosine phosphorylation in cultured cells. Large scale proteome analysis combining the separation of proteins on two-dimensional gel with protein identifications with MALDI-TOF MS made it possible to identify 93 phosphorylated proteins by heat shock in RIF-1 and TR-RIF-1 cells. This is the first report that 93 proteins

| Table IV—continued |
|---------------------|
| **Proteomic Analysis of Heat Shock Signaling Pathways** |
| **-kinase** | **-SH2/PTB** |
| PDGF-FR | Itk | InsR | Lck | FGF-R | Akt | EGFR | Src | PLCγ | Ctk | Grb2 | Akt | Fyn | NCK | FGFR | Lck | SHIP | Src | Shc | p65 | PDZ |
| 63 phosphoglycerate kinase | 161 |
| 64 phosphoglycerate kinase 1 | 161 |
| 65 aldolase 1, A isofrom | |
| 66 phospholipid transferase | 364 |
| 67 MAPK | 305 |
| 68 MAPK | 305 |
| 69 MAPK | 305 |
| 70 GAPDH | 92 | 49 |
| 71 GAPDH | 92 | 49 |
| 72 hspRNP A2/B1 | 123, 285 | 123, 285 | 123, 285 | 254, 275 |
| 73 hspRNP A2/B1 | 254, 275 |
| 74 annexin II, lipocortin II | |
| 75 TIS | 186 | 136 |
| 76 lactate dehydrogenase A | 20 | 20 |
| 77 creatin kinase, M chain | 121 | 132 |
| 78 TIS | 205 |
| 79 hspRNP A2/B1 | 254, 275 |
| 80 voltage dependent anion channel 1 | |
| 81 G protein beta subunit like | |
| 82 L-34 protein (AA 1-264) | 121 | 132 |
| 83 NcN | |
| 84 pyruvate kinase isozyme M2 | |
| 85 pyruvate kinase isozyme M2 | |
| 86 RNA-binding protein alpha-CF | |
| 87 aldolase reductase | 134 | 148 | 148 | 139 |
| 88 voltage dependent anion channel 2 | |
| 89 Unidentified | |
| 90 glutathione-S-transferase homolog | 224 |
| 91 similar to phosphoglycerate mutase 1 | |
| 92 triphosphatase isomerase | |
| 93 triphosphatase isomerase | |

**Number of predicted proteins**: 12 | 7 | 6 | 7 | 5 | 4 | 5 | 13 | 9 | 7 | 9 | 8 | 6 | 7 | 6 | 5 | 6 | 6 | 1
having various functions are responsive to phosphorylation by heat shock. Eighty one of 93 proteins were identified. Identification of 64 distinct proteins from 81 spots shows the existence of post-translational modifications and alternative splicing in addition to phosphorylation by heat shock. Of these, 21 proteins were reported previously as phosphorylated by various signals (see references in Table III) and 43 are newly identified as phosphorylated by heat shock. In some cases, the amount of protein was too small to be identified from the available sequence data base for the mouse genome. Thus there may well be other novel proteins that have not yet been registered in the protein data base.

These studies further show that kinetic analysis of the phosphorylation can reveal the possible process by which a protein may act in heat shock response which includes a transient immediate blockage of protein synthesis, protein synthesis recovery (Hsps first and then normal proteins after heat shock), and transient induction of thermotolerance. Major differences between thermotolerant TR-RIF-1 cells and control cells include less sensitivity to heat shock and faster recovery after heat shock in TR-RIF-1 cells. Activation of SAPK/JNK, a marker of stress, in TR-RIF-1 cells, by same amount of heat shock, was less than that in control RIF-1 cells (28) because of the insensitivity of thermotolerance cells. The discovery of Hsp and normal protein synthesis in TR-RIF-1 cells was much faster than in control cells as shown in Fig. 1B. Although the rate and extent of heat shock response are different in RIF-1 and TR-RIF-1 cells, the heat shock pathways seem to be the same.

We were able to sort the 81 proteins identified into three groups based on the kinetic analysis of phosphorylation by heat shock. The first group of proteins showed increased basal phosphorylations in TR-RIF-1 cells (without heat shock) over RIF-1 cells and a significant increase in phosphorylation after heat shock only in RIF-1 cells. This suggests that the phosphorylation of the first group of proteins is required for the maintenance of thermotolerance. Mild heat shock to control RIF-1 cells induced transient thermotolerance after 24 h of recovery, which is consistent with phosphorylation status of the first group of proteins in RIF-1 cells after 24 h of recovery after heat shock. This group contains chaperones that are known to be involved in thermotolerance (Hsp70 (2 spots), HSC70, and chaperonin containing TCP-1 e-subunit), and also transcription- and translation-related proteins (L-protein and RNA binding protein α-CP1), oxidoreduction-related proteins (non-selenium glutathione peroxidase and glutathione S-transferase homolog), signaling molecules (CPP32, transforming growth factor-β receptor-binding protein and B-cell receptor-associated protein 32), energy metabolism-related enzymes (pyruvate kinase M2 (3 spots), triosephosphate isomerase, and GAPDH), cytoskeletal proteins (tropomyosin 5 and capping protein β-subunit 2), and 2 putative and 3 unidentified proteins. Newly registered proteins involved in thermotolerance are mainly phosphorylated in response to heat shock. This is consistent with the finding that heat shock induces protein synthesis blockage and recovery (28), and cytoskeletal protein collapse and recovery (48) during recovery after heat shock. Intermediate filament vimentin modifications were identified in response to heat shock previously (48). Translocations of vimentin by various stresses were reported (49, 50).

The broad spectrum of protein phosphorylations observed during recovery after heat shock warrant investigations of the cellular processes in stress-induced cell death and thermotolerance. However, it was impossible to examine many of the individual phosphorylated proteins experimentally. We employed three computer-assisted programs to predict the phosphorylation sites, possible PTKs involved, and binding proteins to be phosphorylated residues. Although each program has different stringency, some predictions common to two of the programs were noted. The results are consistent with the previous findings for phosphorylation sites: Tyr-306 in hnRNP H (spot 11 and 12), tyrosine phosphorylation sites of Hsc70 (spot 1, 2), aldolase A isoform (spot 65), and lactate dehydrogenase A (spot 76). ScanSite predictions allowed us to identify the possible PTKs phosphorylating target proteins by heat shock. Although EGFR PTK phosphorylation sites were predicted in 4 proteins (heat shock protein 70 cognate (spots 1 and 2), Hsp70, aldolase reductase) in this study, the results were in agreement with previous findings (14). c-Src activated in response to heat shock (14) may phosphorylate 5 substrate proteins (hnRNP F, 47-kDa heat shock protein, hnRNP A2/B1 (spots 72, 73, and 79), voltage-dependent anion channel 1) as predicted in this study. Tyrosine phosphorylation recognition motif containing molecules such as Grb2-SH2 and Shc-SH2 were predicted in 10 proteins (phosphoglycerate kinase (spots 63 and 64), MAP kinase kinase (spots 67–69), hnRNP A2/B1 (spots 72, 73, and 79), topoisomerase inhibitor-suppressed (spot 78), similar to phosphoglycerate mutase 1) and 6 proteins (heat shock protein 70 cognate (spots 1 and 2), Hsp70, L-protein (spots 55 and 56), aldolase A (spot 65)), respectively. The activation of the Shc-Grb2 pathway by heat shock was reported previously (43). It appears that PTKs or phosphotyrosine recognition motifs that have been predicted in this study are likely to be involved in activated signal pathways by heat shock.

In summary, proteomic analysis combined with two-dimensional gel, Western blotting and mass spectrometry are powerful tools for globally identifying the key molecules in heat shock signaling. This is the first comprehensive study to report on molecular phenomena in heat shock responses by combining high throughput proteomic analysis with kinetic studies and computer-assisted methodology. Further identification of all molecular pathways involved in heat shock should provide a better understanding of heat-induced signaling pathways and the cellular mechanisms underlying thermotolerance.

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