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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.

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)[1] 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 thermotolerant 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-phos-

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[1] 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."

photyrosine antibody, and mass spectrometry. Kinetic analysis of protein phosphorylation during recovery after heat shock allowed us to develop information on the relationship between phosphorylated proteins and their possible functions in heat shock response. We sorted the phosphorylated proteins identified into three groups based on the kinetic analysis. Computer-assisted predictions of phosphorylation sites, the PTKs possibly involved, and the proteins that possibly bind to these phosphorylated motifs have been performed to understand the large amount of information. This is a new trial to obtain comprehensive understanding in the signaling pathways activated in response to heat shock.

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% CO_2, 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 [^{35}S]Methionine Pulse Labeling—Patterns of cellular protein synthesis after heat shock were examined by pulse labeling with [^{35}S]methionine (1 μCi/ml) 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 $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 20 μM/ml leupeptin, 5 mM Na_3VO_4, 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 pellet was resuspended in half-packed cell volume of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 20 μM/ml leupeptin, 5 mM Na_3VO_4, 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 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 EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 20 μM/ml leupeptin, 5 mM Na_3VO_4, 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 protein 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 pepstatin A, 10 μg/ml leupeptin, 1 mM EDTA, 10 mM Na_3VO_4, 10 mM NaF and electrofocused in 7-cm Immobiline™ DryStrips (pH 4–7, 6–11) with the Amersham Biosciences IPGphor. The following focusing protocol was used: 50 μA per strip at 20 °C; 1) rehydration for 16 h; 2) 500 V for 1 h (step and hold); 3) 1000 V for 1 h (step and hold); and 4) 8000 V for 3 or 9 h (step and hold). After electrofocusing, the strips were shaken for 15 min with equilibration buffer (1.5 M Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml dithiothreitol) and loaded onto Bio-Rad minigel two-dimensional SDS-PAGE. Proteins from SDS-PAGE were stained with Coomassie Blue or Silver or electroblotted onto a nitrocellulose membrane. The blot was incubated with anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology, Inc.) at a 1:1000 dilution in PBST at 4 °C overnight. The protein-antibody complexes were visualized with horseradish peroxidase-goat anti-mouse immunoglobulin G conjugate at a 1:2000 dilution. The blots were incubated for 1 min in the ECL plus kit (Amersham Biosciences) solution and exposed to x-ray film (Hyperfilm, Amersham Biosciences). Images were scanned using a LAS-1000S CCD-based camera and semi-quantitatively analyzed using the software package ImageMaster two-dimensional software (Amersham Biosciences). Each sample was run in triplicate together with internal standards of phosphotyrosine of known molecular weights (Upstate Biotechnology, Inc.) and the ratio of intensity between sample and internal standard was used for the quantitative analysis.

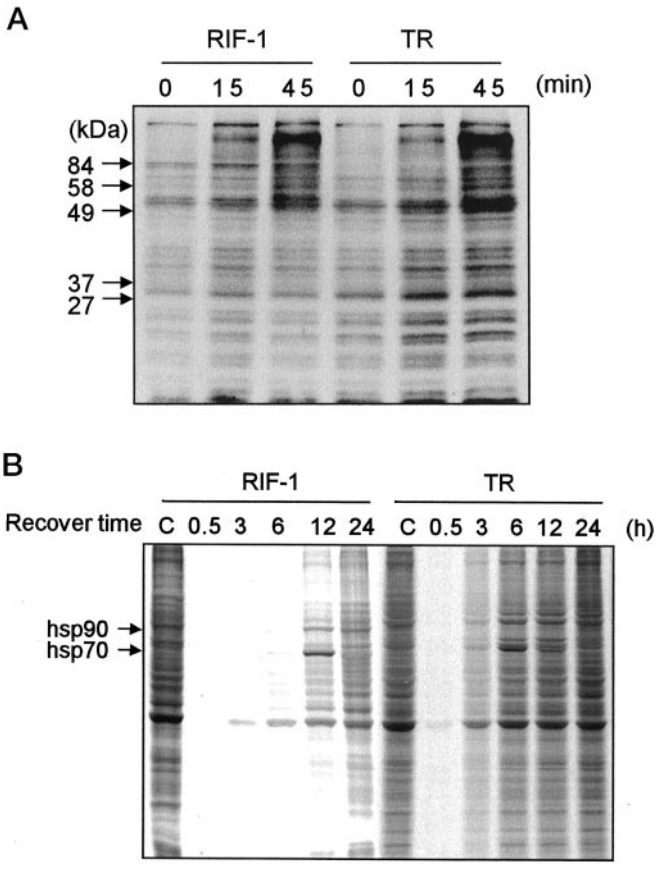

FIG. 1. **Heat shock effects on tyrosine phosphorylation and synthesis of proteins.** *A*, RIF-1 and TR-RIF-1 cells were treated with heat shock at 45 °C for 0, 15, and 45 min. Cells were separated on SDS-PAGE and immunostained with phosphotyrosine-specific monoclonal antibody. Proteins were visualized by chemiluminescence and the image was read by LAS-1000S. *B*, RIF-1 and TR-RIF-1 cells were treated with heat shock at 45 °C for 30 min and recovered for 0.5, 3, 6, 12, and 24 h. At the indicated times, cells were pulse-labeled with 1 μCi/ml [^{35}S]methionine in methionine-free RPMI 1640 media for 1 h, and the same amounts of proteins were separated on 10% SDS-PAGE and autoradiographed by BAS2500. *C* indicates control.

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 some modifications (26, 27). The gel spots were excised with a scalpel, crushed, and destained by washing with 25 mM ammonium bicarbonate, 50% acetonitrile. In the case of silver-stained gel, it is destained by washing with 15 mM $K_4Fe(CN)_6$, 50 mM sodium thiosulfate prior to crushing the gel. The gels were dehydrated by addition of acetonitrile, rehydrated by adding 10–20 μl of 25 mM ammonium bicarbonate with 10 ng/μl of sequencing grade trypsin (Promega), and incubated at 37 °C for 12–15 h. Peptides were extracted by adding 30 μl of solution containing 60% acetonitrile, 0.1% trifluoroacetic acid. The extraction was repeated three times and completed by adding 20 μl of acetonitrile. The extracted solutions were pooled and evaporated to dryness in a SpeedVac vacuum centrifuge. Samples were reconstituted in 10 μl of 0.1% trifluoroacetic acid and treated with ZipTips containing C18 resin (Millipore) according to the manufacturer's instructions. The washed peptides were eluted with saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.1% trifluoroacetic acid).

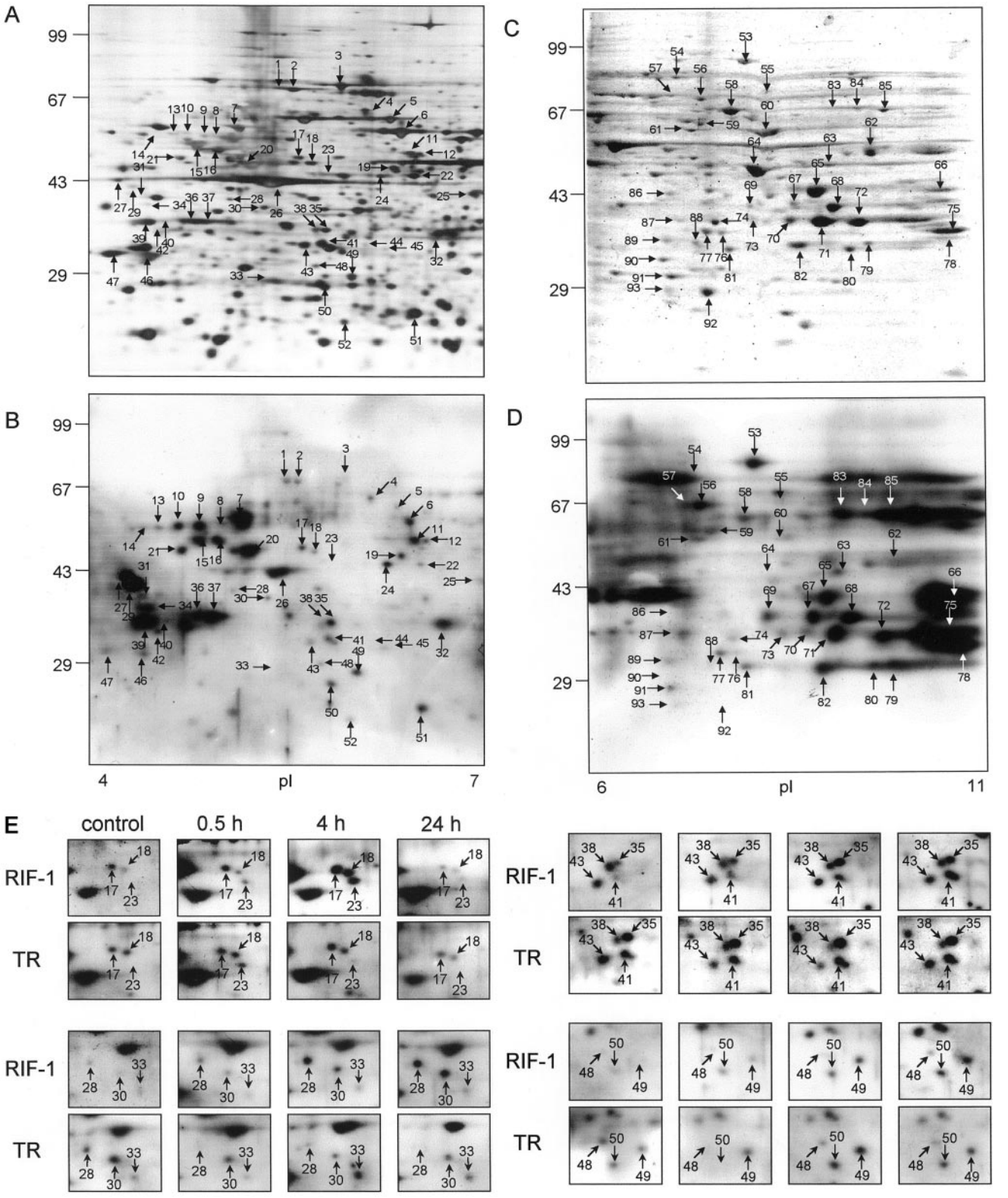

FIG. 2. **Two-dimensional gel images visualized by silver staining, Coomassie Blue staining, and immunostaining.** RIF-1 and TR-RIF-1 cells were analyzed by two-dimensional gel and visualized by staining. One of the best images was presented. *A*, two-dimensional silver-stained gel map of pI range 4–7. *B*, two-dimensional phosphotyrosine immuno-stained gel map of pI range 4–7. *C*, two-dimensional Coomassie Blue-stained gel map of pI range 6–11. *D*, two-dimensional phosphotyrosine immuno-stained gel map of pI range 6–11. Numbered spots were excised and analyzed by in-gel trypsin digestion and MALDI-TOF MS. *E*, immunoblots of proteins detected with anti-phosphotyrosine antibodies of heat shock-treated cells. Western blot analysis was carried out after two-dimensional gel electrophoresis of the heat shock treated or not treated total proteins. Analyzed spots are indicated by *arrows*, and the numbers match the numbers on *A* and *B*.

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-

TABLE I
A list of identified proteins

Spot No.	Identified protein	NCBI Accession No.	Mass	pI	Coverage
			Da		%
	Proteins that act as chaperones/mediators of protein folding				
1	Heat shock protein 70 cognate	309319	70,837.6	5.38	34
2	Heat shock protein 70 cognate	309319	70,837.6	5.38	32
3	HSP70	3986773	70,079.7	5.53	40
5	CCT (chaperonin containing TCP-1) ε-subunit	468550	59,624.6	5.72	54
6	Similar to ER-60 protease	13096984	56,678.8	5.88	58
62	47-kDa heat shock protein	303678	46,534.0	8.88	23
	Proteins that function in transcription and translation				
4	Heat shock transcription factor 2	51448	58,155.6	4.77	6
11	hnRNP H	2253041	49,199.9	5.89	14
12	Murin homolog of human ftp-3 (hnRNP H)	1666700	49,280.0	5.89	31
20	Ribonucleoprotein F	17390408	45,730.0	5.31	29
36	Nucleolar protein	200011	32,560.3	4.62	26
37	Eukaryotic translation elongation factor 1-δ	10442752	31,293.3	4.91	25
47	Eukaryotic translation elongation factor 1 β2	13278099	24,693.9	4.53	34
48	Similar to replication protein A2	13435424	29,430.4	5.47	23
55	L-protein (*Homo sapiens*)	32356	60,187.8	6.65	16
56	L-protein (*Homo sapiens*)	32356	60,187.8	6.65	13
72	hnRNP A2/B1	3329498	35,993.3	8.67	37
73	hnRNP A2/B1	3329498	35,993.3	8.67	45
75	TIS	1711240	34,196.5	9.27	48
78	TIS	1711242	28,897.8	8.97	29
79	hnRNP A2/B1	3329498	35,993.3	8.67	45
83	NonO	1083440	54,599.3	8.86	29
86	RNA-binding protein α-CP1	5805273	37,498.2	6.66	44
	Proteins that act in amino acid biosynthesis				
22	Ornithine-oxo-acid aminotransferase	53459	48,355.0	6.19	23
59	Inosine-5-monophosphate dehydrogenase 2	124427	55,785.5	6.84	25
61	Glutamate dehydrogenase	51082	61,337.3	8.05	30
66	Precytosolic aspartate aminotransferase	309110	47,411.8	9.13	12
	Proteins that are related to oxidation or reduction				
51	Nonselenium glutathione peroxidase	2072655	24,870.9	5.71	59
90	Glutathione *S*-transferase homolog	2393724	27,497.9	6.91	54
	Proteins that compose channels				
80	Voltage-dependent anion channel 1	6755963	30,755.6	8.62	76
88	Voltage-dependent anion channel 2	6755965	31,733.0	7.44	14
	Proteins that act signaling molecules				
24	Annexin A7	14290464	49,909.7	5.91	22
32	CPP32	2317650	31,475.1	6.45	31
35	TGF-β receptor-binding protein	6014677	36,461.1	5.38	25
50	Prohibitin or B-cell receptor associated protein 32	541732	29,820.3	5.57	36
67	MAP kinase kinase	1495702	37,432.6	7.00	32
68	MAP kinase kinase	1495702	37,432.6	7.00	26
69	MAP kinase kinase	1495702	37,432.6	7.00	24
74	Annexin II, lipocortin II	6996913	38,676.4	7.55	28
81	G protein β subunit-like	2137308	35,018.9	8.08	16
82	L-34 protein (amino acids 1–264)	52851	27,428.9	8.58	24
	Proteins that function in cell motility or structure				
7	Vimentin	2078001	51,564.7	4.96	51
8	Vimentin	2078001	51,564.7	4.96	58
9	Vimentin	2078001	51,564.7	4.96	15
15	β-Tubulin	55048	49,640.3	4.78	45
17	BAF53a	4001805	47,430.4	5.40	21
21	Vimentin	2078001	51,564.7	4.96	33
26	γ-Actin	809561	41,019.4	5.56	52
29	β-Tropomyosin	50190	32,945.0	4.61	15
33	Capping protein α1 subunit	595917	32,751.8	5.34	20
38	α-Tubulin Isotype M-α-6	202217	49,909.8	4.96	24
39	α-Tropomyosin	509182	32,680.8	4.69	27
40	α-Tropomyosin	509182	32,680.8	4.69	17
43	β-Tubulin	55048	49,671.3	4.78	36
46	Tropomyosin 5	54912	29,020.9	4.75	34
49	Capping protein β-subunit, isoform 2	500749	30,629.0	5.69	31
54	Lamin A	346883	74,185.1	6.41	38

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-

TABLE I—*continued*

Spot No.	Identified protein	NCBI Accession No.	Mass	pI	Coverage
			Da		%
Proteins that act in energy metabolism					
16	ATP synthase β-subunit	2623222	56,380.1	5.14	38
19	Proteasome 26S subunit, ATPase 2	13529470	48,648.3	5.72	38
28	Galactokinase	8650486	42,295.6	5.17	23
53	Similar to mitochondrial aconitase	13435538	85,464.2	8.08	29
58	Pyruvate kinase, M2 isozyme	2506796	57,887.3	7.17	35
63	Phosphoglycerate kinase	202423	44,536.8	7.54	28
64	Phosphoglycerate kinase	202423	44,536.8	7.54	29
65	Aldolase 1, A isoform	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	538135	36,498.9	7.61	36
77	Creatine kinase, M chain	125306	43,045.3	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	27
87	Aldolase reductase	3046247	35,718.5	6.71	20
91	Similar to phosphoglycerate mutase 1	12805529	28,832.2	6.68	59
92	Triosephosphate isomerase	54855	26,695.9	6.90	40
93	Triosephosphate isomerase	54855	26,695.9	6.90	34
Miscellaneous					
18	Putative	12850298	52,769.2	5.75	21
27	Reticulocalbin	220582	38,113.4	4.70	24
30	RIKEN cDNA 2410174K12 gene	14318755	38,159.2	5.32	29
41	Putative	12848170	38,937.5	6.41	36
52	β-Proteasome subunit	1762779	29,116.4	5.47	34
60	RIKEN cDNA 2700043D08 gene	13435984	55,761.2	8.72	35
10, 13, 14, 23, 25, 31, 34, 42, 44, 45, 57, 89[a]					

[a] These spot numbers are unidentified.

rylated proteins were detected with anti-phosphotyrosine antibody 4G10 (Fig. 1*A*). Tyrosine phosphorylations increased in a dose-dependent manner in both RIF-1 and TR-RIF-1 cells. Some of the phosphorylated proteins were common to the two cell lines and some were different.

Next we carried out metabolic labeling of the newly synthesized proteins to determine the kinetics of protein synthesis after heat shock in both cells. Although heat shock at 45 °C for 45 min showed more dramatic increase of tyrosine phosphorylation as shown in Fig. 1*A*, the cells for this study were exposed to heat shock at 45 °C for only 30 min because heat shock of RIF-1 cells at 45 °C for 45 min caused the severe cell death (28). Protein synthesis was monitored by [^{35}S]methionine pulse labeling during recovery after heat shock. Cells were exposed to heat shock at 45 °C for 30 min, allowed to recover for various lengths of time, and then labeled with [^{35}S]methionine for 1 h at 37 °C to measure the protein synthesis rates. Equal amounts of proteins were applied on each lane of SDS-PAGE. As shown in Fig. 1*B*, heat shock in both cell lines immediately blocked total protein synthesis which was gradually restored with Hsp synthesis first and followed by total synthesis. Recovery rate of protein synthesis in TR-RIF-1 cells was much faster than in RIF-1 cells and both cell lines recovered to normal after 24 h of recovery. Longer exposure to heat shock at 45 °C blocked protein synthesis to a greater degree and resulted in slower recovery or cell death (data not shown). This suggests that it is important to examine the kinetics of tyrosine phosphorylation level of each protein during recovery after heat shock rather than at a fixed time point.

Identification of Phosphorylated Proteins—To determine the tyrosine-phosphorylated proteins involved in heat shock response, proteome analysis was performed with cells exposed to heat shock and recovered for various times. Both RIF-1 and TR-RIF-1 cells were subjected to heat shock at 45 °C for 30 min and recovered at 37 °C for 0, 4, and 24 h. Cellular proteins harvested at each time point were separated on two sets of two-dimensional gel in two pI ranges, 4–7 and 6–11. The gels were visualized by Coomassie Blue or Silver staining or were blotted to NC membrane and detected with immunostaining using anti-phosphotyrosine monoclonal antibody and ECL chemiluminescence detection kit (Fig. 2). The immunostained NC membranes were exposed to x-ray film for various times to find the linear range depending on the concentrations of the phosphorylated proteins. The film images were scanned using ImageMaster LabScan version 3.00 software connected to Umax scanner. We found 93 phosphorylated proteins containing both basally phosphorylated proteins and heat shock-induced 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 6–11. Immunostained spots detected in any one of the immunoblot analyses were overlaid and are depicted by *numbered arrows* in the silver-stained gel (Fig. 2*A*) and Coomassie Blue-stained gel (Fig. 2*C*). The corresponding immunostained gels were shown in Fig. 2, *B* and *D*. Examples of heat shock-induced tyrosine phosphorylation changes detected with immunostaining are shown in Fig. 2*E*.

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

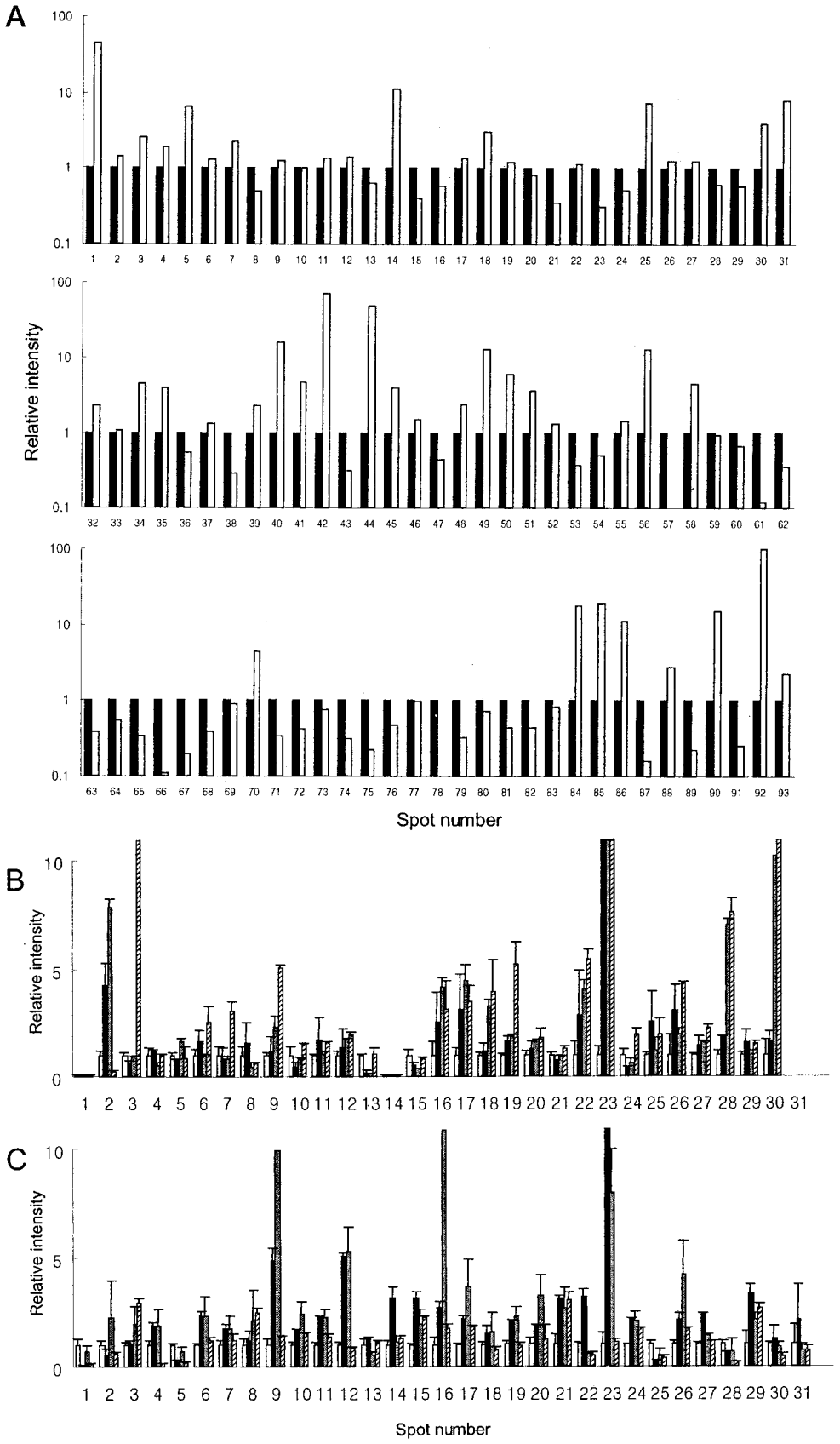

FIG. 3. **Quantification of protein tyrosine phosphorylation.** *A*, comparison of basal phosphorylations in control RIF-1 cells and thermotolerant TR-RIF-1 cells. Immunostained spots with phosphotyrosine antibody in unstressed RIF-1 (*black bars*) and TR-RIF-1 (*white bars*) cells were quantified, and 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; *gray bars*, 4 h recovered cells after heat shock; *hatched bars*, 24 h recovered cells. *Bars* more than 10-fold were abbreviated.

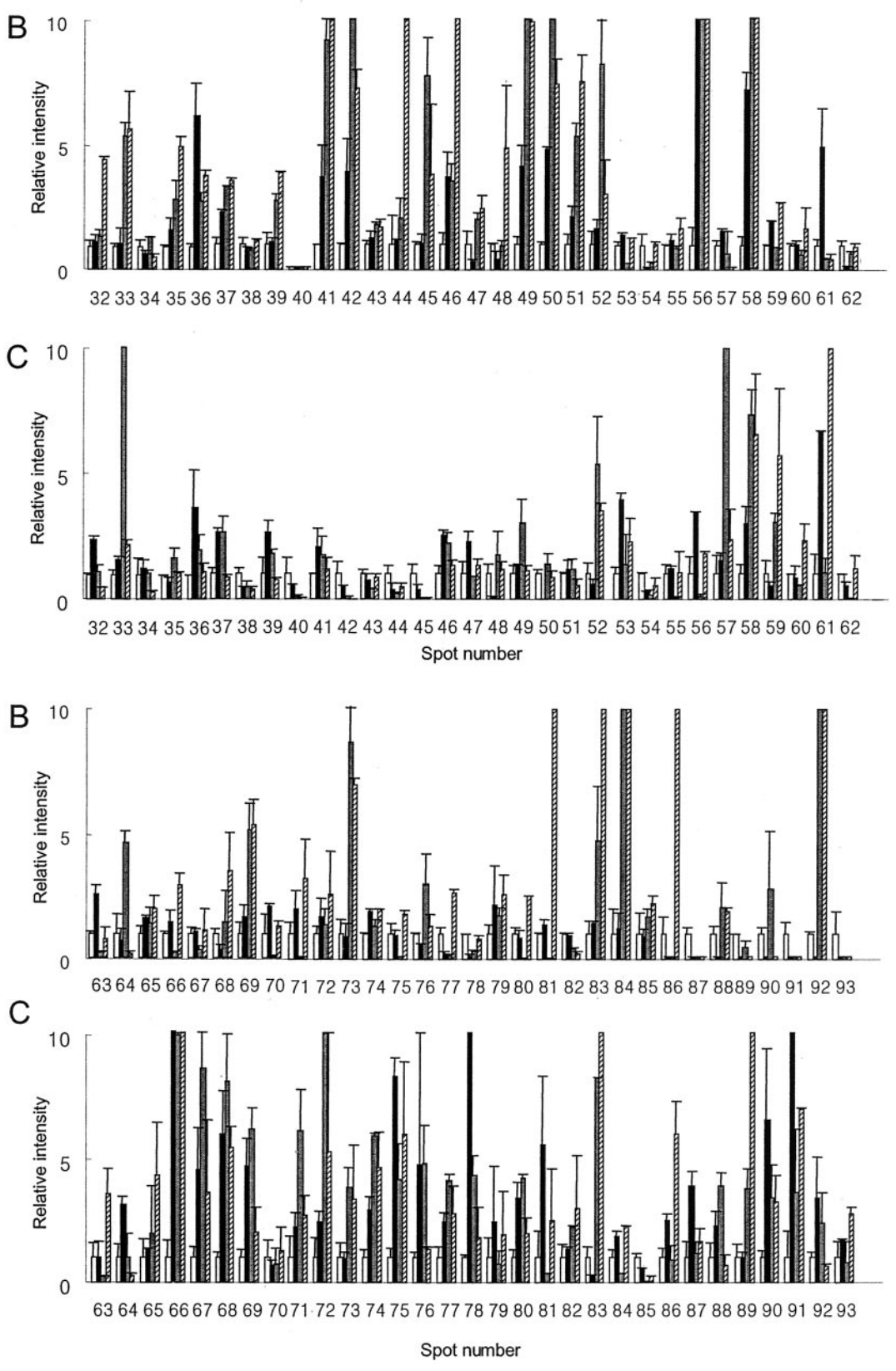

FIG. 3—*continued*

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

TR/RIF, tyrosine phosphorylation level of control 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

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)ε-subunit	++	1	1	+	1	1	+	+	0
Proteins that function in transcription and translation										
56	L-protein (H. *sapiens*)	+++	1	+++	+++	+++	1	+	0	+
86	RNA-binding protein α-CP1	+++	1	0	0	++++	1	+	1	++
Proteins that are related to oxidation or reduction										
51	Nonselenium glutathione peroxidase	++	1	+	++	++	1	1	1	1
90	Glutathione *S*-transferase homolog	+++	1	0	+	0	1	++	+	+
Proteins that compose channels										
88	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	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	++	+	++
78	TIS	− − −	1	0	0	1	1	+++	+	+
Proteins that act in amino acid biosynthesis										
61	Glutamate dehydrogenase	− −	1	++	0	0	1	++	1	+++
66	Precytosolic aspartate aminotransferase	− − −	1	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	++	++	++
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	+	+	+

tra did not match with mouse protein database. Different spots including spots 1 and 2 (heat shock protein 70 cognate), 11 and 12 (hnRNP H), 55 and 56 (L-protein), 67, 68, and 69 (MAP kinase kinase), 72, 73, and 79 (hnRNP A2/B1), 75 and 78 (topoisomerase inhibitor-suppressed), 7, 9, and 21 (vimentin), 39 and 40 (α-tropomyosin), 15 and 43 (β-tubulin), 63 and 64 (phosphoglycerate kinase), 70 and 71 (GAPDH), 58, 84, and 85 (pyruvate kinase isozyme M2), and 92 and 93 (triosephosphate isomerase) turned out to be the same proteins possibly due to modifications such as phosphorylation or degradation. Spot 18, 30, 41, and 60 were identified as putative proteins or unnamed proteins with known sequences. Blast search for the homologous sequence proteins showed that these have human homologous proteins, ubiquitol-cytochrome *c* reductase core I protein, suppressor of G2 allele of skp1 homolog, pyruvate dehydrogenase, and mitochondrial serine hydroxymethyltransferase, respectively. These results suggest that proteins having various cellular functions were tyrosine-phosphorylated during heat shock response. This type of massive proteomic analytical approach which agrees with previous results may provide clues to the understanding of the heat shock phenomena.

Heat Shock Changes in the Levels of Protein Phosphorylation—To determine the possible molecules involved in heat shock responses 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

TABLE II—*continued*

Spot No.	Identified protein	TR/RIF	RIF-1				TR-RIF-1			
			c	Imm	4h	24h	c	Imm	4h	24h
Proteins that act in energy metabolism										
16	ATP synthase β-subunit	−	1	+	+	+	1	+	+++	+
53	Similar to mitochondrial aconitase	−	1	1	0	1	1	+	1	+
71	GAPDH	−	1	+	0	+	1	+	++	+
76	Lactate dehydrogenase A	−	1	1	+	1	1	++	++	1
87	Aldolase reductase	−−	1	0	0	0	1	+	1	+
91	Similar to phosphoglycerate mutase 1	−	1	0	0	0	1	+++	+	++
Miscellaneous										
57	Unidentified	−−−	1	+	1	0	1	+	+++	+
89	Unidentified	−−	1	0	1	0	1	1	+	+++
PROTEIN FAMILY 3										
Proteins that act as chaperones/mediators of protein folding										
6	Similar to ER-60 protease	=	1	+	+	+	1	+	+	1
Proteins that function in transcription and translation										
4	Heat shock transcription factor 2	+	1	1	1	1	1	+	+	0
11	hnRNP H	=	1	+	+	+	1	+	+	1
12	Murin homolog of human ftp-3 (hnRNP H)	=	1	1	+	+	1	++	++	1
20	Ribonucleoprotein F	=	1	1	+	+	1	+	+	1
36	Nucleolar protein	−	1	++	+	+	1	+	+	1
37	Eukaryotic translation elongation factor1-δ	+	1	+	+	+	1	+	+	1
47	Eukaryotic translation elongation factor1 β2	−	1	1	+	+	1	+	1	1
48	Similar to replication protein A2	+	1	1	1	++	1	0	+	1
55	L-protein (H. *sapiens*)	=	1	1	1	+	1	1	0	1
73	hnRNP A2/B1	=	1	1	++	++	1	1	+	+
79	hnRNP A2/B1	−	1	+	+	+	1	+	1	+
83	NonO	=	1	1	++	+++	1	0	++	+++
Proteins that act in amino acid biosynthesis										
22	Ornithine oxo-acid aminotransferase	=	1	+	+	++	1	+	1	1
59	Inosine-5-monophosphate dehydrogenase 2	=	1	+	+	+	1	1	+	++
Proteins that act as signaling molecules										
69	MAP kinase kinase	=	1	+	++	++	1	++	++	+
81	G protein β-subunit-like	−	1	1	0	+++	1	++	0	+
Proteins that function in cell motility or structure										
7	Vimentin	+	1	1	1	+	1	+	+	1
8	Vimentin	−	1	+	1	1	1	1	+	+
9	Vimentin	=	1	+	+	++	1	++	+++	1
17	BAF53a	=	1	+	+	+	1	+	+	+
26	γ-Actin	=	1	+	+	+	1	+	+	+
29	β-Tropomyosin	=	1	+	1	1	1	+	+	+
33	Capping protein α-1 subunit	=	1	1	++	++	1	+	+++	+
39	α-Tropomyosin	+	1	1	+	+	1	+	+	+
Proteins that act in energy metabolism										
19	Proteasome 26 S subunit, ATPase 2	=	1	+	+	++	1	+	+	1
28	Galactokinase	=	1	+	++	++	1	1	1	0
63	Phosphoglycerate kinase	−	1	+	0	1	1	1	0	+
64	Phosphoglycerate kinase 1	−	1	1	++	1	1	+	1	0
65	Aldolase 1, A isoform	−	1	+	+	1	1	1	+	+
77	Creatine kinase, M chain	=	1	0	0	+	1	+	+	+
93	Triosephosphate isomerase	+	1	0	0	0	1	+	1	+
Miscellaneous										
10	Unidentified	=	1	0	1	+	1	+	+	1
14	Unidentified	++	1	1	1	1	1	+	1	1
18	Putative	+	1	1	+	+	1	1	+	1
23	Unidentified	−	1	+++	+++	+++	1	+++	++	1
27	Reticulocalbin	=	1	1	+	+	1	+	1	1
52	β-Proteasome subunit	+	1	+	++	+	1	1	++	+
60	RIKEN cDNA 2700043D08 gene	−	1	1	1	+	1	1	1	+

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-

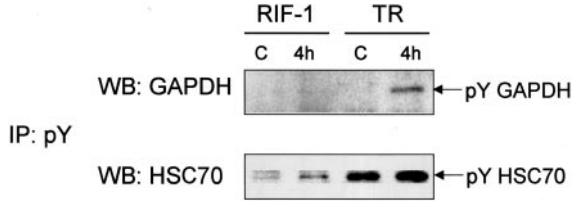

FIG. 4. **Heat shock-induced tyrosine phosphorylation of GAPDH and heat shock protein 70 cognate.** RIF-1 and TR-RIF-1 cells were either left untreated or heat shock-treated at 45 °C for 30 min and recovered for 4 h at 37 °C. Cleared cell lysates and nuclear fractions were put together and immunoprecipitated with anti-phosphotyrosine antibodies, washed, and resolved by SDS-PAGE. The gel was transferred to NC membrane and then probed with polyclonal anti-GAPDH or monoclonal anti-HSP70 antibody. Immunocomplexes were detected by ECL solution kit and read by LAS-1000 S.

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.
Proteins that act as chaperones/mediators of protein folding				
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) ε-subunit	12, 137, 274	503	
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		
Proteins that function in transcription and translation				
4	Heat shock transcription factor 2	279, 293, 362	249	
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	
20	ribonucleoprotein F	11, 194, 210, 253, 266, 276, 306, 372, 414	306	
36	Nucleolar protein			
37	Eukaryotic translation elongation factor 1-δ	18, 26	18	
47	Eukaryotic translation elongation factor 1 β 2	24, 28, 213		
48	Similar to replication protein A2	14, 20, 125, 256		
55	L-protein (*Homo sapiens*)	61, 132, 173, 181, 254, 293, 297, 302, 332, 344, 399	254	
56	L-protein (*Homo sapiens*)	61, 132, 173, 181, 254, 293, 297, 302, 332, 344, 399	254	
72	hnRNP A2/B1	123, 222, 232, 264, 275, 288		29
73	hnRNP A2/B1	123, 222, 232, 264, 275, 288		29
75	TIS	128, 244, 253, 262		
78	TIS	128, 244, 260, 289, 314		
79	hnRNP A2/B1	123, 222, 232, 264, 275, 288		29
83	NonO	160, 269		35
86	RNA-binding protein α-CP1		207	
Proteins that act in amino acid biosynthesis				
22	Ornithine-oxo-acid aminotransferase	45, 50, 126, 158, 194, 209, 245, 299		52
59	Inosine-5-monophosphate dehydrogenase 2	4, 41, 258, 289, 294, 430		
61	Glutamate dehydrogenase	193, 240, 289, 429, 539	464	
66	Precytosolic aspartate aminotransferase	316, 401, 420	96, 401	
Proteins that are related to oxidation or reduction				
51	Nonselenium glutathione peroxidase	217		
90	Glutathione *S*-transferase homolog	97, 108, 160, 174	108	
Proteins that compose channels				
80	Voltage-dependent anion channel 1	7, 62, 118		
88	Voltage-dependent anion channel 2	19, 74		
Proteins that act as signaling molecules				
24	Annexin A7	6, 358, 377, 401, 402		
32	CPP32	32, 41, 128, 147, 195, 274	32	
35	TGF-β receptor-binding protein	139, 300, 308, 318		
50	Prohibitin or B-cell receptor-associated protein (BAP) 32			
67	MAP kinase kinase	339	305	
68	MAP kinase kinase	339	305	
69	MAP kinase kinase	339	305	
74	Annexin II, lipocortin II	24, 30, 151, 188, 275, 327		31–34, 54
81	G protein β-subunit-like	52	52	
82	L-34 protein (AA 1–264)			

ance, we therefore compared the degree of protein tyrosine phosphorylation in RIF-1 cells and TR-RIF-1 cells at basal level (Fig. 3*A*) and we examined the kinetics of tyrosine phosphorylation of each protein during recovery after heat shock (Fig. 3*B*, *C*). We chose the following three recovery time points based on protein synthesis profiles shown in Fig. 1*B*: (*a*) immediately after heat shock when protein synthesis is completely blocked in both of RIF-1 and TR-RIF-1 cell lines; (*b*) after 4 h of recovery time when TR-RIF-1 cells are restarting protein synthesis including Hsp synthesis but not RIF-1 cells; and (*c*) after 24 h recovery when both of cell lines are recovered, protein synthesis returned to normal, and transient thermotolerant state even in RIF-1 is sustained. The degree of phosphorylations of each spot was quantified and normalized over standard markers and control cells.

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) 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 second group of proteins showed higher basal tyrosine phosphorylation levels in RIF-1 cells than in TR-RIF-1 cells (Table II, PROTEIN FAMILY 2). 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-

TABLE III—*continued*

Spot No.	Identified protein	NetPhos prediction site	ScanProsite prediction site	Ref.
Proteins that function in cell motility or structure				
7	Vimentin	10, 33, 256, 271		14, 52, 55
8	Vimentin	10, 33, 256, 271		14, 52, 55
9	Vimentin	10, 33, 256, 271		36, 52, 55
15	β-Tubulin	59, 106, 200, 222, 310, 425		52
17	BAF53a	69, 144, 253, 274, 287, 416		
21	Vimentin	11, 30, 53, 276, 291		36, 52, 55
26	γ-Actin	46, 191, 211, 233, 287, 355	191, 211	55
29	β-Tropomyosin	60, 162, 214, 221		
33	Capping protein α1 subunit	134, 197, 237, 245		
38	α-Tubulin isotype M-α-6	210, 224, 399, 432, 449	172	
39	α-Tropomyosin	60, 162, 214, 221, 261, 267		
40	α-Tropomyosin	60, 162, 214, 221, 261, 267		
43	β-Tubulin	59, 106, 200, 222, 310, 425		52
46	Tropomyosin 5	126, 178, 185		
49	Capping protein β-subunit, isoform 2	71, 107, 111		
54	Lamin A	359	267	35, 52
Proteins that act in energy metabolism				
16	ATP synthase β-subunit	247, 418	395	52
19	Proteasome 26 S subunit, ATPase 2	4, 111, 174, 429	147	
28	Galactokinase	47, 300, 318	312	
53	Similar to mitochondrial aconitase	42, 306, 390, 432, 544, 665, 715	432	
58	Pyruvate kinase, M2 Isozyme	161, 175, 370	148, 390	
63	Phosphoglycerate kinase	76, 161, 196, 324		
64	Phosphoglycerate kinase 1	76, 161, 196, 324		
65	Aldolase 1, A isoform	85, 328, 343, 364	223	36
70	GAPDH	47, 138, 253, 316, 318	328	30
71	GAPDH	47, 138, 253, 316, 318	328	30
76	Lactate dehydrogenase A	83, 239	239	30, 37, 53
77	Creatine kinase, M chain	20, 82, 125, 173, 279		
84	Pyruvate kinase isozyme M2	161, 175, 370	148, 390	
85	Pyruvate kinase isozyme M2	161, 175, 370	148, 390	
87	Aldolase reductase	122	40	
91	Similar to phosphoglycerate mutase 1	133, 142	92	30, 37, 53
92	Triosephosphate isomerase	48		53
93	Triosephosphate isomerase	48		53
Miscellaneous				
18	Putative	131, 219, 348, 450		
27	Reticulocalbin	111, 134, 222		
30	RIKEN cDNA 2410174K12 gene	95, 145, 189, 254		
41	Putative	67, 132		
52	β-Proteasome subunit	102, 169		52
60	RIKEN cDNA 2700043D08 gene	100, 234, 270, 309	309	

teins in TR-RIF-1 cells may make TR-RIF-1 cells like RIF-1 cells and trigger heat shock signaling pathway as RIF-1 cells do. This group includes 3 proteins functioning in transcription and translation, 2 proteins related to amino acid biosynthesis, 1 voltage-dependent anion channel, 5 signaling molecules, 2 cytoskeletal proteins, 6 energy metabolism-related proteins, and 2 unidentified proteins.

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. This 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 7 putative proteins or unidentified proteins.

Before the quantification, we expected that most of the proteins would show increased levels of protein tyrosine phosphorylation (Fig. 1*A*). 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. 3*B* 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. 3*A*. In addition,

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.

	-kinase								-SH2/PTB													
	PDGFR	Itk	InsR	Lck	FGFR	Abl	EGFR	Src	PLC$_\gamma$	Crk	Grb2	Abl	Fyn	NCK	FGFR	Lck	SHIP	Itk	Src	Shc	p85	PDZ
1 heat shock protein 70 cognate	67						67													314		
2 heat shock protein 70 cognate	67						67													314		
3 HSP70	41						41													149		
4 heat shock transcription factor 2	293			293	293													293				
5 CCT (chaperonin containing TCP-1) epsilon subunit						454				115				115	264							
6 similar to ER-60 protease																						
7 vimentin																						
8 vimentin																						
9 vimentin																						
10 unidentified																						
11 hnRNP H		306		306	306				180, 376	210												
12 murin homolog of human ftp-3 (hnRNP H)	376	306		306	306				180, 376	210				210								
13 unidentified																						
14 unidentified																						
15 beta-tubulin																						
16 ATP synthase beta-subunit																					247	
17 BAF53a																						
18 putative																						
19 proteasome 26s subunit, ATPase 2																	192					
20 hnRNP F (rat)		306			246, 306			306				194	243									
21 vimentin										210												
22 ornithine-oxo-acid aminotransferase	42		126														209					
23 unidentified																						
24 annexin A7										32												
25 unidentified																						
26 gamma actin												233										
27 reticulocalbin																						
28 galactokinase																						
29 beta-tropomyosin													162		162	162			162			
30 RIKEN cDNA 2410174K12 gene									336						336							
31 unidentified																						
32 CPP32																						
33 capping protein alpha 1 subunit																						
34 unidentified																						
35 TGF-beta receptor binding protein	158		158																			
36 nucleolar protein																						
37 eukaryotic translation elongation factor 1-delta													26								126	
38 alpha tubulin isotype M-alpha-6	449	449	499										432		432, 449	432, 449		449	432			
39 alpha tropomyosin													162		162	162			162			
40 alpha tropomyosin													162		162	162			162			
41 putative																						
42 unidentified																						
43 beta-tubulin																						
44 unidentified																						
45 unidentified																						
46 tropomyosin 5													126		126	126			126			
47 eukaryotic translation elongation factor 1 beta 2																						
48 similar to replication protein A2																						
49 capping protein beta subunit, isoform 2														79								
50 prohibitin or B-cell associated protein (BAP) 32														114								
51 nonselenium glutathione peroxidase																						
52 beta proteosome subunit																					169	
53 similar to mitochondrial aconitase						544			88	544		544	390	544			45, 71	544				
54 lamin A												481										
55 L-protein (homo sapiens)						357			124	86, 327		86, 279, 357		86, 279				327		198		
56 L-protein (homo sapiens)						357			124	86, 327		86, 279, 357		86 279				327		198		
57 unidentified																						
58 pyruvate kinase, M2 isozyme																						
59 inosine-5-monophosphate dehydrogenase 2					400																	
60 RIKEN cDNA 2700043D08 gene																						
61 glutamate dehydrogenase			289																			
62 47 kDa heat shock protein								370														

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—*continued*

		-kinase							-SH2/PTB												
	PDGFR	Itk	InsR	Lck	FGFR	Abl	EGFR	Src	PLCγ	Crk	Grb2	Abl	Fyn	NCK	FGFR	Lck	SHIP	Itk	Src	Shc	p85	PDZ
63 phosphoglycerate kinase											161											
64 phosphoglycerate kinase 1											161											
65 aldolase 1, A isoform																				364		
66 precytosolic aspartate aminotransferase									75			316										
67 MAPK																			305			
68 MAPK																			305			
69 MAPK																			305			
70 GAPDH		92	49																			
71 GAPDH		92	49																			
72 hnRNP A2/B1	228			123, 288				123	341		264, 275											
73 hnRNP A2/B1	228			123, 288				123	341		264, 275											
74 annexin II, lipocortin II																188			316			
75 TIS																						
76 lactate dehydrogenase A																						
77 creatin kinase, M chain	20				20				279			140										
78 TIS											266											
79 hnRNP A2/B1	288			123, 288				123	341		264, 275											
80 voltage dependent anion channel 1				195																		
81 G protein beta subunit like																						
82 L-34 protein (AA 1-264)						121								132								
83 NonO																						
84 pyruvate kinase isozyme M2																						
85 pyruvate kinase isozyme M2																						
86 RNA-binding protein alpha-CP1																						
87 aldolase reductase		134			148		148		139													
88 voltage dependent anion channel 2																						
89 Unidentified																						
90 glutathione-S-transferase homolog									224													238
91 similar to phosphoglycerate mutase 1										119	133	119		119								
92 triosphosphate isomerase																						
93 triosphosphate isomerase																						
Number of predicted proteins	12	7	6	7	7	5	4	5	13	9	7	9	8	9	7	6	3	5	6	6	3	1

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)*X*(2)(DE)*X*(3)Y or (RK)*X*(3)(DE)*X*(2)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 isoform (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

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 thermotolerant cells. The recovery of Hsp and normal protein synthesis in TR-RIF-1 cells was much faster than in control cells as shown in Fig. 1*B*. 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 ϵ-subunit), and also transcription- and translation-related proteins (L-protein and RNA binding protein α-CP1), oxidoreduction-related proteins (nonselenium 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 reducing enzymes and key enzymes of the glycolytic pathway including pyruvate kinase M2 and triosephosphate isomerase. The relationship between phosphorylation of these proteins and thermotolerance needs to be established in further studies.

The second group of proteins showed increased phosphorylation in control RIF-1 cells without heat shock, over TR-RIF-1 cells, and a significant increase in phosphorylation by heat shock appearing only in TR-RIF-1 cells. This suggests that phosphorylation of the second group of proteins may be required for the signaling pathway in response to heat shock. Phosphorylated proteins in RIF-1 cells easily respond to heat shock. On the other hand, the reduced phosphorylation in TR-RIF-1 cells may induce the insensitivity and inhibit response to heat shock as shown previously (28). For example, reduced phosphorylations of MAP kinase kinase in TR-RIF-1 cells may cause less activation of MAPK in response to heat shock than RIF-1 cells (data not shown). These studies have identified 20 proteins that are less phosphorylated in thermotolerant cells as heat shock signaling molecules.

The third group of proteins are phosphorylated by heat shock in both RIF-1 and TR-RIF-1 cells, although the extent and kinetics of phosphorylations in response to heat shock were different. These proteins may directly respond to heat shock and may be involved in heat shock signaling pathways regardless of thermotolerance. This group of proteins may be functioning in transcription and translation (12 proteins) and in cell motility and structure (8 proteins). 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 only 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 definition of the 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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Proteomic Analysis of Protein Phosphorylations in Heat Shock Response and Thermotolerance

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