Thioredoxin Is Transcriptionally Induced upon Activation of Heat Shock Factor 2

Sirpa Leppä‡§, Lila Pirkkala‡§**, Sek C. Chow‡†, John E. Eriksson‡, and Lea Sistonen‡§§

From the ‡Turku Centre for Biotechnology, University of Turku, Åbo Akademi University, FIN-20521 Turku, Finland, the §Department of Biology, Åbo Akademi University, BioCity, FIN-20521 Turku, Finland, and the ¶Center for Mechanism of Human Toxicity, University of Leicester, Leicester LE1 9HN, United Kingdom

Heat shock gene expression is differentially regulated in cells exposed to stress stimuli and in cells undergoing processes of differentiation and development. Regulation of the classical heat shock response is mediated by heat shock factor 1 (HSF1), whereas heat shock factor 2 (HSF2) is activated in certain differentiating cells, for example during hemin-mediated differentiation of human K562 erythroleukemia cells. Hence, the signaling pathways leading to induction of heat shock gene expression upon different stimuli are likely to be distinct. We have used RNA arbitrarily primed polymerase chain reaction to identify genes that are differentially regulated upon activation of HSF1 and HSF2. In this study, we report that thioredoxin (TRX) expression is induced in K562 cells in response to hemin in an HSF2-dependent manner. Increased TRX expression was primarily detected on the transcriptional level, subsequently leading to elevated TRX mRNA and protein levels. Hemin treatment caused no reduction in cellular glutathione concentrations, indicating that the increased TRX expression was not due to oxidative stress. Studies using cell lines where overexpression of the HSF2-β isoform represses HSF2 activation implied that active HSF2 is required for transcriptional induction of TRX. Unlike HSF2, activation of HSF1 did not induce TRX expression. Taken together, our results suggest that HSF1 and HSF2 may regulate distinct target genes, and activation of HSF2 could be involved in the regulation of TRX expression during hemin-mediated differentiation of K562 cells.

The heat shock response, characterized by induction of heat shock gene expression leading to increased synthesis of heat shock proteins, is a ubiquitous cellular response to elevated temperatures as well as other forms of stress (for review see Refs. 1–3). In addition, increased expression of heat shock genes is induced under several unstressed conditions, including certain stages of embryonic development and differentiation (for review see Ref. 4). The regulation of heat shock gene expression in eukaryotes is mediated at the transcriptional level by pre-existing transcriptional activators, heat shock factors (HSFs),‡ that bind to regulatory heat shock elements present in the promoter region of all heat shock genes (for review see Refs. 5–7). In Drosophila, only one HSF has been identified, and this factor has both developmental and stress-responsive functions (8). In mammals, two differentially regulated transcriptional activators exist; HSF1 is the functional homologue of the general HSF that is activated by classical stress stimuli, whereas HSF2 is activated during hemin-mediated differentiation of human K562 erythroleukemia cells (9–12). In addition, HSF2 is constitutively active in mouse embryonal carcinoma cells and during mouse embryogenesis and spermatogenesis (13–16), suggesting that HSF2 is involved in the regulation of heat shock gene expression in cells undergoing processes of differentiation and development. Yet the signals and signaling pathways leading to activation of HSF2 are not known. Furthermore, the observations that HSF2 recognizes heat shock elements slightly differently and activates heat shock gene expression less efficiently than HSF1 (9, 17, 18) have raised the possibility that HSF2 may have other target genes apart from the known heat shock genes.

Recently, the existence of two distinct HSF2 isoforms, HSF2-α and HSF2-β, has been demonstrated in mouse tissues (19, 20). The smaller isoform, HSF2-β, is generated from the transcript by an additional splice, resulting in the deletion of an 18-amino acid sequence relative to the larger HSF2-α isoform (20). Because the expression of HSF2-α and HSF2-β isoforms varies between different tissues, it is plausible that these two proteins are functionally distinct. In fact, HSF2-α has been shown to be a more potent transcriptional activator than HSF2-β, as analyzed by transient transfection in NIH 3T3 cells (20). Furthermore, the hemin-mediated acquisition of HSF2 DNA binding activity and transcriptional induction of heat shock gene expression are repressed in cells overexpressing HSF2-β, as has been demonstrated by using stably transfected K562 cell clones that express various levels of mouse HSF2 isoforms (21). These results imply that regulation of HSF2 activity is complex and involves differential expression of HSF2 isoforms presumably in a tissue- and/or cell type-dependent manner.

RNA arbitrarily primed PCR (RAP-PCR) has been shown to be a powerful method for identifying transcripts that are differentially expressed during the cell cycle, tissue and organ development, and tumorigenesis (for review see Ref. 22). In an attempt to identify genes that are differentially regulated during the HSF1-mediated heat shock response and upon activation of HSF2, we have used the RAP-PCR method described by Ralph and co-workers (23). In this study, we report that TRX is

‡ This work was supported by funds from the Academy of Finland, the Cell Signaling Program of the Åbo Akademi University, Sigrid Juselius Foundation, the Finnish Cultural Foundation, and the Finnish Cancer Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ These authors contributed equally to this work.
¶ Present address: European Molecular Biology Laboratory, P.O. Box 10,2209, D-69012 Heidelberg, Germany.
** Graduate student in the Turku Graduate School of Biomedical Sciences.
*** To whom correspondence should be addressed: Turku Centre for Biotechnology, P.O. Box 123, FIN-20521 Turku, Finland. Tel.: 358-2-333-8028; Fax: 358-2-333-8000; E-mail: lea.sistonen@btk.utu.fi.

The abbreviations used are: HSF, heat shock transcription factor; GSH, glutathione; PCR, polymerase chain reaction; RAP-PCR, RNA arbitrarily primed PCR; TRX, thioredoxin.
transcriptionally induced in concert with activation of HSF2 in hemin-treated K562 cells but not upon activation of HSF1. This observation is of particular interest concerning the differential regulation of gene expression by distinct HSFs, apart from their established function in the transcriptional induction of heat shock genes.

EXPERIMENTAL PROCEDURES

Cell Culture, Hemin Treatment, and Heat Shock Conditions—Human K562 erythroleukemia cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 units/ml), and 2 mM glutamine in a humidified 5% CO2 atmosphere at 37 °C. K562 cells stably overexpressing HSF2-α and HSF2-β isoforms (21) were maintained in RPMI 1640 medium containing G418 (500 µg/ml; Life Technologies, Inc.). Prior to exposure to hemin or heat shock treatments, 5 × 10^5 cells were plated in medium without G418. Hemin (Aldrich) was added to a final concentration of 20 or 30 µM, and heat shock was performed in a 42 °C water bath.

RAP-PCR and Isolation of cDNA Clones—Cytoplasmic RNA from hemin- or heat shock-treated cells was isolated and treated with DNase I. RAP-PCR was performed as described by Khalil and co-workers (23). Briefly, 50 ng of RNA was reverse transcribed with an arbitrary primer (5'-CCCATACGGCTTATCCTG-3'). The same primer was used to amplify the cDNA in the presence of [α-32P]dCTP. The extended DNA products were separated on a 6% polyacrylamide sequencing-type gel and visualized by autoradiography. PCR products representing the transcripts of interest were isolated from the gel, reamplified by PCR, cloned into pGEM-T (Promega), and sequenced using the Sequenase reagent kit (U. S. Biochemical Corp.).

Northern Blot Analysis and Nuclear Run-on Assay—For Northern blot analysis, 10 µg of total RNA isolated from hemin- or heat shock-treated cells was separated on a 1% agarose-formaldehyde gel, transferred to nylon membrane (Hybond-N, Amersham Corp.), and hybridized at 65 °C with a [α-32P]dCTP (50 µCi, 3000 Ci/mmole; ICN)-labeled 500-base pair insert of human TRX and plasmids specific for human hsp70 (24), human hsp90/89a (25), and human β-actin (26). Following hybridization, filters were washed with 0.1 × SSC-0.1% SDS at 65 °C and visualized by autoradiography. The intensities of radioactive signals were quantitated using phosphoimaging (Bio-Rad), and the obtained values were normalized against β-actin.

Nuclear run-on transcription reactions were performed with equal numbers of nuclei (7 × 10^6) isolated from hemin- or heat shock-treated cells in the presence of 100 µCi of [α-32P]dUTP (3000 Ci/mmole; Amer sham Corp.) as described previously (27). Radiolabeled RNA was hybridized to nitrocellulose-immobilized probes for TRX, hsp70, hsp90/89a, β-actin, and SpD-910. Northern signals were visualized by autoradiography. The intensities of radioactive signals were quantitated using phosphoimaging, and the obtained values were normalized against β-actin.

Preparation of Whole Cell Extracts and Immunoblotting—Whole cell extracts were prepared from hemin- or heat shock-treated cells as described previously (28). 12 µg of protein was separated on a 15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell) using a Bio-Rad semidry transfer apparatus. TRX was detected by a rabbit polyclonal anti-TRX antibody (StressGen). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega and Amersham Corp. The signals were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

Measurements of Cellular Glutathione (GSH)—K562 cells were seeded at 1 × 10^5 cells/3.5-cm-diameter plate prior to addition of hemin to a final concentration of 30 µM. At indicated time points, cells were collected by centrifugation and washed once with phosphate-buffered saline, and the cell pellet was lysed with 500 µl of 6% trichloroacetic acid. After centrifugation of the precipitated proteins, the cellular GSH concentrations were determined from triplicate samples as thiols soluble in trichloroacetic acid by colorimetric analysis as described previously (30). Cells incubated for 1 h with 100 µM menadione (Sigma) were used as a positive control for oxidative stress.

RESULTS

Increased Expression of TRX in Hemin-treated K562 Cells—To assess the changes in gene expression occurring in concert with induction of heat shock gene expression in hemin-treated K562 cells, cytoplasmic RNA was isolated from hemin-treated and heat-shocked cells and analyzed by RAP-PCR. The expression patterns were compared with control mRNAs isolated from untreated cells. As shown in Fig. 1A, several transcripts were differentially expressed upon exposure to heat shock or hemin treatment. To determine whether the PCR products corresponded to inducible mRNAs, cDNAs were recovered, amplified, and subcloned. Subsequent sequence analysis using the GenBank® data base revealed that one of the hemin-inducible cDNAs was identical to the sequence encoding human TRX (31). Northern analysis confirmed that a 0.5-kilobase mRNA coding for TRX was specifically induced upon hemin treatment in a time-dependent manner (Fig. 1B). The mRNA levels of TRX increased gradually, reaching maximal levels at 16–24 h and declining back to basal levels by 72 h (Fig. 1B). In comparison, expression of the major heat shock genes was analyzed from the same samples, showing marked increases of the hsp70 mRNA levels upon exposure to heat shock or hemin, whereas the hsp90 mRNA levels were only moderately increased (Fig. 1B). Subsequently, TRX protein levels in untreated, heat-shocked, and hemin-treated K562 cells were analyzed by Western blotting using a TRX-specific antibody. Following increased amounts of TRX mRNA, the levels of TRX protein increased within 16 h of hemin treatment (Fig. 1C). Accumulation of the inducible Hsp70 protein both in heat-shocked and hemin-treated cells and the constitutive expression of Hsc70 were consistent with our previous results (21).

Transcriptional Induction of TRX—To examine whether induction of TRX expression upon hemin treatment was regulated on the transcriptional level, nuclear run-on assay was performed with nuclei isolated from heat-shocked and hemin-treated K562 cells. Consistent with the results shown in Fig. 1, heat shock did not induce transcription of the TRX gene (Fig. 2). In contrast, transcription of TRX was enhanced 2–3-fold upon exposure to hemin for 16–24 h. The kinetics of the transcriptional induction of TRX was similar to that of hsp70 and hsp90. Furthermore, transcription of both hsp70 and hsp90 was induced to a significantly higher level in response to heat shock (70- and 7-fold, respectively) than upon hemin treatment (2–3-fold), which is in agreement with previous studies, showing that HSF1 is a more potent transcriptional activator of hsp70 and hsp90 gene expression than HSF2 (9, 18). Interestingly, transcription of hsp27 gene, which was induced 6-fold by heat shock, was not markedly affected by hemin treatment. In conclusion, these results demonstrate that induction of TRX expression in hemin-treated K562 cells is primarily regulated on the transcriptional level.

Activation of HSF2 Is Required for Induction of TRX Gene Expression—Because induction of TRX expression occurred in close correlation with the activation of HSF2, we next examined whether HSF2 could be involved in the hemin-mediated induction of TRX. For this purpose, we used transfected K562 cell clones constitutively overexpressing either HSF2-α or HSF2-β. Our previous studies have shown that upon exposure of the HSF2-β-overexpressing cells to hemin, the acquisition of HSF2 DNA binding and transcriptional activity was suppressed, whereas HSF2 was prominently activated in cells overexpressing HSF2-α, indicating that HSF2-β may function as a negative regulator of HSF2 activity during hemin-mediated erythroid differentiation of K562 cells (21). Mock transfected cells (vector) and cells transfected either with HSF2-α (2α-F4 and 2α-C7) or HSF2-β (2β-C8 and 2β-D5) were exposed to heat shock or hemin, nuclei and total RNA were isolated and analyzed by nuclear run-on assay and Northern blotting, re-
Fig. 1. Increased expression of TRX in hemin-treated K562 cells. A, RAP-PCR analysis of mRNAs from K562 cells. 50 ng of cytoplasmic RNA isolated from control (C1 and C2), hemin-treated (HE, 20 or 30 μM for 16 h), and heat-shocked (HS, 1 h at 42 °C) K562 cells was analyzed using RAP-PCR in the presence of [α-32P]dCTP. Samples were separated on a 6% urea-polyacrylamide gel electrophoresis. The asterisks indicate some differentially amplified products between control and treated K562 cells. The product indicated by an arrow was subsequently identified as human TRX, Hsp70, and Hsc70. B, expression levels of TRX (thioredoxin), hsp70, hsp90, hsp27, and β-actin genes were isolated from control (lane C), hemin-treated (HE, 30 μM for 16 and 24 h), and heat-shocked (HS, 1 h at 42 °C) K562 cells, and radiolabeled transcripts were hybridized to immobilized DNA probes. Note that the weak signal of β-actin in the heat-shocked sample is due to down-regulation by heat stress as has been previously shown (53). C, accumulation of TRX protein upon hemin treatment. Whole cell extracts (12 μg) were separated on a 15% SDS-polyacrylamide gel electrophoresis and Western immunoblotted using antibodies against TRX, Hsp70, and Hsc70.

Fig. 2. Hemin treatment induces TRX on the transcriptional level in K562 cells. Transcription rates of TRX (thioredoxin), hsp70, hsp90, hsp27, and β-actin genes were analyzed by nuclear run-on assay. Nuclei were isolated from control (lane C), hemin-treated (HE, 30 μM for 16 and 24 h), and heat-shocked (HS, 1 h at 42 °C) K562 cells, and radiolabeled transcripts were hybridized to immobilized DNA probes. Note that the weak signal of β-actin in the heat-shocked sample is due to down-regulation by heat stress as has been previously shown (53).

Induction of TRX Is Not Caused by Oxidative Stress in Hemin-treated K562 Cells—TRX is a widely distributed redox protein that is known to play an important role in cellular responses against oxidative damage (for review see Refs. 32–34). Because induction of TRX could be a result of an altered cellular redox state, we wanted to determine whether possible redox effects could be underlying the observed increase in TRX expression upon hemin treatment. For this purpose, K562 cells were treated with hemin up to 72 h, and the concentrations of intracellular GSH were analyzed. Menadione, a pro-oxidant that causes oxidative stress by generation of superoxide radicals through redox cycling thereby reducing intracellular GSH levels (35, 36), was used as a positive control. As shown in Fig. 4, unlike menadione, hemin caused no decrease but rather an increase in the GSH levels. Hence, hemin treatment does not cause oxidative stress in K562 cells. Elevated GSH levels were detected gradually after 16 h, and the maximum 4-fold increase occurred after 2 days of exposure to hemin, after which the GSH levels reached a plateau. A similar increase in the GSH levels was observed in HSF2-α- and HSF2-β-overexpressing cells (data not shown), indicating that GSH synthesis does not depend on activation of HSF2 in K562 cells.

DISCUSSION

Previous studies have indicated that the signaling pathways of mammalian HSFs are distinct (9, 10, 12, 13, 16, 17, 37). Moreover, the Drosophila HSF has recently been shown to have multiple functions because mutational analysis has revealed that HSF in Drosophila is essential not only for the heat shock response but is also required during oogenesis and larval development (8). Interestingly, the developmental functions of Drosophila HSF appear not to be mediated through the induction of heat shock genes (8). Furthermore, recent studies on
TRX is a widely distributed redox-active protein that regulates intracellular redox-dependent processes, including transcriptional activity, proliferation, and apoptosis (Ref. 29 and references therein). Hence, TRX is associated with a number of crucial functions other than acting as an antioxidant. Interestingly, TRX, which has been identified also as adult T cell leukemia-derived factor, has been reported to be produced by many human T cell lymphotrophic virus-1-transformed T cells and Epstein-Barr virus-transformed B cells (for review see Ref. 38). Adult T cell leukemia-derived factor/TRX has been shown to act as a growth promoter of both normal and tumor cells (39–41) and to operate in synergism with interleukin-1 and interleukin-2 (42). Furthermore, many human cancers overexpress TRX (43, 44). These effects may all stem from its roles in transcriptional activities of transcription factors, including NF-κB and AP-1 (45–47). Recent studies have shown that TRX enhances the DNA binding activity of AP-1 through a direct association with the redox/repair protein Ref-1 (48, 49). Our results showing that TRX levels in K562 cells are elevated in concert with the differentiation-related processes induced by hemin occurring in the absence of oxidative stress favor the idea that TRX would function as an important effector protein in cell growth and differentiation. Furthermore, our study provides evidence that HSF2 would be an initiating factor for the elevated TRX expression in hemin-treated K562 cells.

The mechanisms regulating TRX gene expression are poorly understood. The available data relate to transcriptional regulation during oxidative stress. A recent report on increased TRX expression following exposure to oxidative agents in Jurkat cells revealed specific DNA binding activities to the TRX promoter sequences that show no homology with any known consensus sequences for DNA-binding factors (50). However, we have no reason to believe that our results would be related to oxidative stress. Using the above-mentioned novel oxidative stress responsive element and nuclear extracts from hemin-treated K562 cells for gel mobility shift assay, we were not able to detect any inducible protein-DNA interactions (data not shown). Furthermore, oxidant-induced heat shock response has been demonstrated to activate HSF1 instead of HSF2 (51). In this study, we could not observe any activation of HSF1. Therefore, the observed HSF2 activation followed by elevated TRX expression appears to be a completely separate process from the oxidant-induced elevation of TRX expression.

Interestingly, the increased expression of TRX was followed by elevated levels of GSH. However, our results show that the kinetics of GSH synthesis is clearly different from that of TRX induction. Whereas the GSH levels increase until 2 days of exposure to hemin, the induction of TRX expression seems to be transient, peaking at 16–24 h (Figs. 1 and 4). This implies that...
hemin-mediated induction of TRX and regulation of the intracellular GSH levels are not directly coupled. Nevertheless, both GSH and TRX will contribute to shifting the intracellular environment toward a more reducing state. Little is known about the roles of GSH in differentiation-related processes. In this regard, K562 cells have previously been reported to synthesize and recycle GSH efficiently, and serum-starved K562 cells greatly increase their reduced GSH in response to growth factors by activating de novo biosynthesis (52).

Although it remains to be shown whether HSF2 is directly involved in the transcriptional regulation of TRX, our results on the transcriptional induction of TRX upon activation of HSF2 point toward a novel regulatory role of HSF2. In addition, this study further reinforces the distinction between the pathways leading to the activation of the mammalian transcription factors HSF1 and HSF2.

Acknowledgments—We thank Garth Powis for kindly providing human TRX antibody, Dirk Bohmann for critical comments on the manuscript, and Helena Saarento for excellent technical assistance.

REFERENCES

1. Lindquist, S., and Craig, E. A. (1988) Annu. Rev. Genet. 22, 631–677
2. Morimoto, R. I., Tissières, A., and Georgopoulos, C. (1990) Stress Proteins in Biology and Medicine, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Feige, U., Morimoto, R. I., Yahara, I., and Polla, B. S. (1996) Stress-inducible Cellular Responses, Birhauser Verlag, Basel
4. Hightower, L., and Nover, L. (1991) Cell. Biol. 5, 2392–2397
5. Lis, J. T., and Wu, C. (1993) J. Biol. Chem. 268, 15293–15298
6. Wollman, E. E., d’Auriol, L., Rimsky, L., Shaw, A., Jacquot, J.-P., Wingfield, P., Graber, P., Dessars, F., Robin, P., Galibert, F., Bertoglio, J., and Fradelizi, D. (1988) J. Biol. Chem. 263, 15056–15152
7. Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966
8. Powis, G., Kirkpatrick, D. L., Angulo, M., Gasdaska, J. R., Broome Powell, M., Salmon, S. E., and Montfort, W. R. (1996) Anti-Cancer Drugs 7, Suppl. 3, 121–126
9. Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., and Orrenius, S. (1982) J. Biol. Chem. 257, 12419–12425
10. Chaput, M., Brygier, J., Lion, Y., and Sels, A. (1983) Biochimie 65, 501–512
11. Nakai, A., Tanabe, M., Kawaoze, Y., Inazawa, J., Morimoto, R. I., and Nagata, K. (1997) Mol. Cell. Biol. 17, 469–481
12. Yodoi, J., and Tursz, T. (1990) Adv. Cancer Res. 57, 381–411
13. Yamauchi, A., Masutani, H., Tagaya, Y., Wakasugi, N., Mitsui, A., Nakamura, H., Inamoto, T., Ozawa, K., and Yodoi, J. (1992) Mol. Immunol. 29, 263–270
14. Ohlberg, J. E., Bergmann, M., Gasdaska, P. Y., and Powis, G. (1994) J. Biol. Chem. 269, 11714–11720
15. Gasdaska, J. R., Bergmann, M., and Powis, G. (1995) Cell Growth Differ. 6, 1643–1650
16. Wasi, P., Tagaya, Y., Wakasugi, H., Mittas, A., Maeda, M., Yodoi, J., and Tursz, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8282–8286
17. Nakamura, H., Masutani, H., Tagaya, Y., Yamauchi, A., Inamoto, T., Nambu, Y., Fujii, S., Ozawa, K., and Yodoi, J. (1992) Cancer Res. 52, 2091–2097
18. Gasdaska, P. Y., Ohlberg, J. E., Cotgreave, I. A., and Powis, G. (1994) Biochem. Biophys. Acta 1218, 292–296
19. Matthews, J. R., Wakasugi, N., Virelizier, J. L., Yodoi, J., and Hay, R. T. (1992) Nucleic Acids Res. 20, 5821–5830
20. Shenkel, H., Klein, M., Erdbrüger, W., Droge, W., and Schulze-Osthoff, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1672–1676
21. Qin, J., Clare, G. M., Kennedy, W. M., Huth, J. R., and Grunenborn, A. M. (1995) Structure 3, 269–277
22. Xanthoudakis, S., and Curran, T. (1992) EMBO J. 11, 653–665
23. Hirota, K., Matsui, M., Iwata, S., Nishiyama, M., Mori, K., and Yodoi, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3633–3638
24. Taniguchi, Y., Taniguchi-Ueda, Y., Mori, K., and Yodoi, J. (1996) Nucleic Acids Res. 24, 2746–2752
25. Jakubovski, J. L., and Sato, Y. (1996) Biochem. J. 318, 187–193
26. Frisch, H., Kennedy, E. J., Chigurupati, R., and Sivarajan, M. (1993) J. Clin. Invest. 92, 2761–2767
27. Abravaya, K., Phillips, B., and Morimoto, R. I. (1991) Mol. Cell. Biol. 11, 586–592

Nucleic Acids Res. 23, 467–474