Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 202-208

THE TYPE II HEMOPEXIN INTERLEUKIN-6 RESPONSE ELEMENT PREDOMINATES THE TRANSCRIPTIONAL REGULATION OF THE HEMOPEXIN ACUTE PHASE RESPONSIVENESS[1]

STEPHAN IMMENSCHUH[2], DE-XIU SONG[3], HIROYUKI SATOH[4] AND URSULA MULLER-EBERHARD[5]

Departments of Pediatrics, Biochemistry and Pharmacology, Cornell University Medical College, New York, NY 10021

Received December 19, 1994

Hemopexin (Hx) is induced during the acute phase response (APR) by the cytokine interleukin (IL)-6. A type II IL-6 response element (RE) of the Hx gene has been characterized recently (J. Biol. Chem. (1994); 269, 12654-12661). To assess Hx gene regulation by other agents, various cytokines and growth factors were tested for their ability to induce Hx in rat hepatoma H-35 cells. IL-6-type cytokines, IL-1ß and TNF-α, in contrast to transforming growth factor-ß (TGF-ß), hepatocyte growth factor and insulin significantly increased Hx gene expression. Chloramphenicol acetyltransferase (CAT) activity in H-35 cells transfected with constructs that contained the 5'-flanking Hx promoter region or multiple copies of the Hx IL-6-RE fused to the CAT gene was upregulated only by IL-6-type cytokines, although to varying degrees. These data indicate that signal transduction pathways mediated by IL-6-type cytokines but not those by IL-1ß and TNF-α converge on the common Hx IL-6-RE.

[1]This work was supported by NIH grant DK 30664 and the Robert Leet and Clara Guthrie Patterson Trust to U.M-E. S.I. was supported by a Postdoctoral Fellowship of the Deutsche Forschungsgemeinschaft (Germany).

[2]Present address: Institut für Klinische Chemie und Pathobiochemie der Justus-Liebig-Universität, Gaffkystr. 11, 35392 Gießen, Germany.

[3]Present address: Department of Pediatrics, Division of Endocrinology, Cornell University Medical College, New York, NY 10021.

[4]Present address: Department of Biomolecular Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274, Japan.

[5]To whom correspondence should be sent.

The abbreviations used are:
APP, acute-phase protein; APR, acute-phase reaction; bp, base pair(s); CAT, chloramphenicol acetyltransferase; Dex, dexamethasone; Hx, hemopexin; IL, interleukin; LIF, leukemia inhibitory factor; RE, response element; TGF-ß, transforming growth factor-ß.

0006-291X/95 $5.00

Hx is a plasma $ß_2$-glycoprotein that binds heme with exceptionally high affinity and can prevent heme-mediated oxidative damage (1, 2). The Hx gene, preferentially expressed in the liver (3, 4), belongs to the group of acute phase proteins (APPs), whose synthesis is regulated by mediators collectively termed cytokines (5, 6).

In this study, an investigation was performed to 1. monitor which cytokines and growth-factors affect the regulatory patterns of Hx protein and mRNA expression, and 2. test for the presence of cis-regulatory elements of cytokines in the promoter 5'-flanking region. Hx was induced significantly by IL-6-type cytokines (IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M), IL-1ß and TNF-α, but not by TGF-ß, hepatocyte growth-factor and insulin. It could be established that the Hx IL-6-RE (7) is the major RE operative in the induction by IL-6-type cytokines.

MATERIALS AND METHODS

Cell Culture and Treatment - H-35 cells (clone T-7-18) (8) were cultured in DMEM; HepG2, Hep3B (9) cells in MEM and Hepa 1-6 cells (10) in RPMI 1640, respectively. All media contained 10% fetal calf serum. Confluent monolayers were incubated in serum-free medium containing 1 μM dexamethasone (Dex) and purified recombinant factors at the following concentrations: COS cell-derived IL-6, 100 ng/ml; CHO cell-derived LIF, 10 ng/ml; IL-11, 100 ng/ml (all from Genetics Institute, Cambridge, MA); TGF-ß, 2 ng/ml; human hepatocyte growth factor, 5 ng/ml; murine TNF-α, 20 ng/ml (all from Genentech, South San Francisco, CA); oncostatin M, 10 ng/ml and human IL-1ß, 10 ng/ml (both from Genzyme, Cambridge, MA). After 24 h equal aliquots of culture media were analyzed for the relative amounts of plasma proteins secreted by rocket immunoelectrophoresis.

RNA Analysis - Total RNA was prepared by the method of Chomczinsky (11). Northern or dot-blot analysis was performed with 15 μg RNA hybridization using a ^{32}P-labeled full-length rat Hx cDNA (3). The hybridization signal was visualized by autoradiography.

Plasmid DNA Transfections - H-35 cells were transfected with plasmid DNA as a DEAE-dextran complex (12). The reporter CAT gene constructs included rat Hx(5xIL-6-RE)CAT (7), pHx(1600)CAT with the 5'-flanking region and 5'-truncated clones pHx(800)CAT and pHx(250)CAT (13). Cytokine treatment and determination of CAT activity was performed as described (7).

RESULTS AND DISCUSSION

Regulation of the rat Hx gene by cytokines and growth factors

A. Hx levels in the media of cultured H-35 cells

The regulation of APP genes can be studied in H-35 hepatoma cells as they retain the qualitative responsiveness to cytokines characteristic of the APR in rat liver (8). In a series of

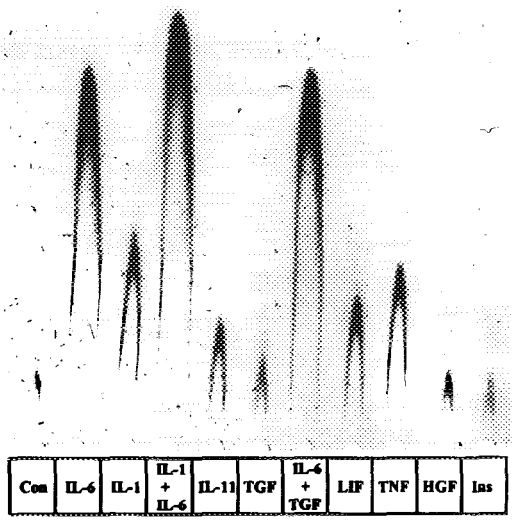

Fig. 1. **Effect of cytokines and growth factors on Hx production.** H-35 cells were grown to confluency and treated for 24 h with serum-free medium containing 1 μM Dex and the indicated cytokines and growth factors. The amount of Hx secreted into the media was analyzed by rocket immunoelectrophoresis using equal volumes of culture media.

preliminary experiments HepG2, Hep3B and mouse Hepa 1-6 cells were tested for their cytokine-specific Hx gene regulation. In contrast to H-35 cells, Hep3B and Hepa 1-6 cells expressed significantly lower levels of Hx mRNA as determined by dot-blot analysis, and in HepG2 cells showed none (data not shown).

Cytokine responsiveness in H-35 cells was evaluated with maximal cytokine concentrations (determined in preliminary experiments). Fig. 1 shows for a representative experiment a comparison of the induction of Hx by IL-6, IL-11, LIF, TNF-α, IL-1ß and by the growth factors TGF-ß, hepatocyte growth factor and insulin in the presence of Dex. IL-6 induced Hx markedly, followed by IL-1ß, TNF-α, LIF and IL-11. Dex showed a marked synergistic effect with cytokines, although it did not alter Hx expression by itself (14). Neither TGF-ß, hepatocyte growth factor nor insulin modulated Hx expression, even in combination with IL-6. The data for TGF-ß differ from previous findings that showed either a synergistic (15) or an inhibitory TGF-ß effect (16) on IL-6 dependent Hx induction in H-35 and Hep3B cells. Mackiewicz et al (17) observed that TGF-ß had no effect on IL-6 induction of haptoglobin or α_1-acid glycoprotein and significantly inhibited that of fibrinogen. The role of TGF-ß on APP gene expression remains to be clarified.

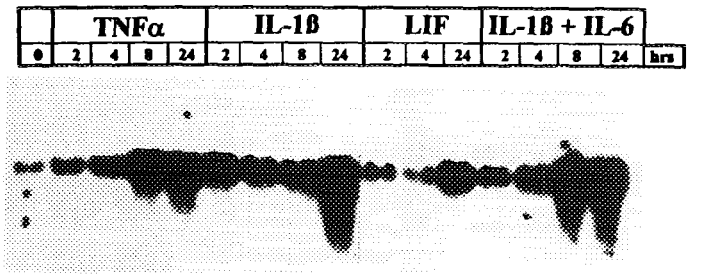

Fig. 2. **Time course of cytokine-specific regulation of Hx mRNA.** H-35 cells grown in 10-cm culture dishes were treated for various lengths of time with serum-free medium with the factors indicated. Total cell RNA was extracted and 15 μg of each preparation was analyzed by Northern blot hybridization for Hx mRNA.

Cytokine-dependent regulation of Hx secretion was compared to that of two other APPs, complement C3 and fibrinogen (data not shown). Complement C3 was stimulated by IL-1ß or IL-1ß + IL-6, but not by IL-6 alone or by one of the growth-factors tested. By contrast, IL-6 induced fibrinogen secretion, which was inhibited by IL-1ß. Thus, the function of cytokines in the regulation of the Hx gene differs from that of other APP genes such as complement C3 and fibrinogen but is similar to that of rat haptoglobin (8).

B. mRNA levels of Hx in cultured H-35 cells

In agreement with the data on Hx secretion, Hx mRNA levels increased during cytokine treatment (Fig. 1). This indicates that cytokines regulate Hx gene expression on a pretranslational level as suggested by the occurence of an enhanced Hx transcription rate in response to IL-6 (7). When compared to IL-6 induction lower Hx mRNA levels were elicited by TNF-α, oncostatin M and LIF, with LIF exhibiting the least responsiveness (data not shown). The time-course of Hx induction (Fig. 2) demonstrated that Hx mRNA levels were upregulated within 2 h, and continued to rise up to 24 h. A slower upregulation of Hx mRNA levels was observed for LIF as compared to those elicited by TNF-α and IL-1ß + IL-6.

Cytokine-mediated regulation of transfected rat Hx gene CAT constructs in H-35 cells

Signaling pathways triggered by various cytokines may converge at the level of APP genes (18, 19). To inquire whether such a mechanism functions in the regulation of the rat Hx gene, H-35

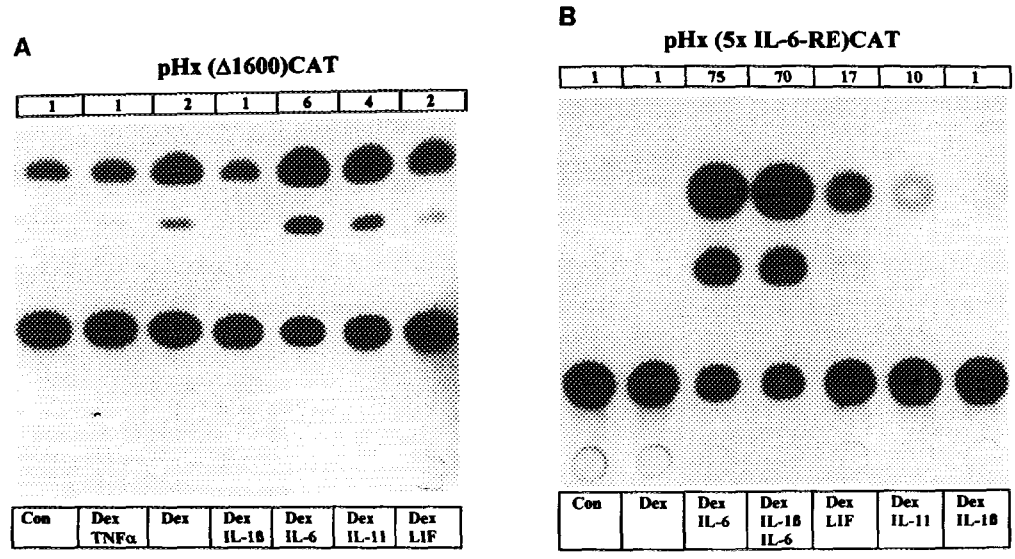

Fig. 3. **Cytokine-dependent regulation of rat pHx(5xIL-6-RE)CAT and pHx(1600)CAT constructs in transiently transfected hepatoma cells.** H-35 cells were transiently transfected with the indicated CAT reporter gene constructs. Cultures were subdivided after a recovery period and treated for 24 h with serum-free medium containing the indicated combinations of cytokines and Dex. The rise in CAT activity in the experiment relative to the control was determined and expressed as the x-fold rise at the top of each panel.

cells were transiently transfected with CAT reporter constructs either containing the Hx promoter plus various lengths of the 5'-flanking region (13) or five copies of the recently demonstrated Hx IL-6-RE (7). The pHx(1600)CAT plasmid was upregulated 2-fold by Dex alone, 6-fold by IL-6 + Dex and 4-fold by IL-11 + Dex (Fig. 3A). Remarkably, IL-1ß as well as TNF-α had an inhibitory effect on CAT-expression levels. The cytokine-dependent induction of the 5'-deleted Hx gene constructs pHx(800)CAT and pHx(250)CAT (data not shown) was similar to that of pHx(1600)CAT and implies that the relevant REs for cytokine responsiveness are located within the first 250bp of the 5'-flanking promoter region. CAT activity of pHx(5xIL-6-RE)CAT was upregulated greatly by IL-6 (75-fold, Fig. 3B). LIF and IL-11 increased CAT activity to a lesser extent (17- and 10-fold, respectively), whereas IL-1ß or Dex had no effect on CAT activity of pHx(5xIL-6-RE)CAT.

The Hx IL-6-RE appears to be the major genetic target for signaling pathways activated by IL-6-type cytokines, which induce to varying degrees (Fig. 3). This finding agrees with previous results on APP gene regulation suggesting that IL-6-type cytokines

utilize at least in part common REs for cytokine-dependent transcriptional regulation (18, 19). The Hx IL-6-RE contains the consensus sequence for type II IL-6-REs (CTGGGAA, 20) that is a nuclear binding site for (the) transcriptional factor(s) IL-6-RE-BP and/or APRF (21, 22). gp130, a non-ligand binding membrane glycoprotein that interacts with cytokine receptors (23), has been ascribed a key role in initiating the signal transduction pathway by which IL-6-type cytokines attach to nuclear target sequences. Alternate signaling mechanisms appear to modulate the cytokine response of Hx (and other APP genes) by REs distant from the Hx IL-6-RE or other cytokine receptor systems.

Although the endogenous Hx gene expression in H-35 cells was induced significantly by IL-1ß and TNF-α (Fig. 1 and 2) none of the CAT constructs was directly upregulated by these cytokines (Fig. 3). This finding concurs with the suggested mediation of TNF-α and IL-1ß to regulate the transcription of APP genes via a receptor system and (a) RE(s) distinct from that for IL-6 regulation (24). It has been reported that C/EBP- and NFkappaB-isoforms are involved in the transcriptional regulation of angiotensinogen, serum amyloid A and complement C3 by IL-1ß and TNF-α (25-28).

The mechanism that mediates the synergistic effect of Dex on cytokine induction is not understood. Two possibilities exist: 1. the glucocorticoid receptor may interact with (an) IL-6 inducible factors(s) to synergistically induce the transcription rate; this was suggested for rat α_1-acid glycoprotein (28); 2. Dex treatment may induce the expression of the IL-6 receptor or the signal transducer gp130 (29, 30). Possibly, a combination of the two mechanisms is responsible for the synergism.

ACKNOWLEDGMENTS

We wish to thank Drs. P. Schendel (Genetics Institute) and G. Wong (Genentech) for providing recombinant cytokines. Furthermore, we thank Dr. H. Baumann for his continuous support during the course of this work.

REFERENCES

1. Muller-Eberhard, U. (1988) in Methods Enzymol. (Disabato, G., ed.), **163**, 536-565, Academic Press, Orlando, Florida
2. Barnard, M.L., Muller-Eberhard, U., and Turrens, J.F. (1993) Biochem. Biophys. Res. Comm. **192**, 82-87
3. Nikkilä, H., Gitlin, J.D. and Muller-Eberhard, U. (1991) Biochemistry 30, 823-829
4. Satoh, H., Nagae, Y., Immenschuh, S., Satoh, T. and Muller-Eberhard, U. (1994) J. Biol. Chem. 269, 6851 -6859

5. Kushner, I. (1982) Ann. N. Y. Acad. Sci. **389**, 39-48
6. Mackiewicz, A., Kushner, I., and Baumann, H. (1993) Acute Phase Proteins : Molecular Biology, Biochemistry and Clinical Applications 1st Ed., CRC Press, Boca Raton, Florida
7. Immenschuh, S., Nagae, Y., Satoh, H., Baumann, H. and Muller-Eberhard, U. (1994) J. Biol. Chem. 269, 12654-12661
8. Baumann, H., Prowse, K.R., Marincovic, S., Won, K.-A., and Jahreis, G.P. (1989) Ann. N.Y. Acad. Sci. 557, 280-295
9. Knowles, B.B., Howe, C.C., and Aden, D.P. (1980) Science 209, 497-499
10. Alam, J., Shibahara, S. and Smith, A. (1989) J. Biol. Chem. 264, 6371-6375
11. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159
12. Lopata, M.A., Cleveland, D.W., and Sollner-Webb, B. (1984) Nucleic Acid Res. 12, 5707-5717
13. Nagae Y., and Muller-Eberhard U. (1992) Biochem. Biophys. Res. Comm. 185, 420-429
14. Baumann, H. and Muller-Eberhard, U. (1987) Biochem. Biophys. Res. Comm. 146, 1218-1226
15. Campos, S.P., Wang, Y., Koj, A. and Baumann, H. (1993) J. Immunol. 151, 7128-7137
16. Morrone, G., Poli, V., Hassan, J.H. and Sorrentino, V. (1992) FEBS Lett. 301, 1-4
17. Mackiewicz, A., Ganapathi, M.K., Schultz, D., Brabenec, A., Weinstein, J., Kelley, M.F. and Kushner, I. (1990) Proc. Natl. Acad. Sci. USA 87, 1491-1495
18. Baumann, H. and Schendel, P. (1991) J. Biol. Chem. 266, 20424-20427
19. Richards, C.D., Brown, T.J., Shoyab, M., Baumann, H. and Gauldie, J. (1992) J. Immunol. 148, 1731-1736
20. Hattori, M., Abraham, L.J., Northemann, W. and Fey, G.H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2364-2368
21. Wegenka, U.M., Buschmann J., Lütticken, C., Heinrich, P.C. and Horn, F. (1993) Mol. Cell. Biol. 13, 276-288
22. Hocke, G.M., Barry, D. and Fey, G.H. (1992) Mol. Cell. Biol. 12, 2282-2294
23. Ip, N.Y., Nye, S.H., Boulton,T.G., Davis, S., Taga, T., Li, Y., Birren, S.J., Yasukawa, K., Kishimoto, T., Anderson, D.J., Stahl, N. and Yancopoulos, G.D. (1992) Cell 69, 1121-1132
24. Akira, S., Hirano, T., Taga, T. and Kishimoto, T. (1990) FASEB J. 4, 2860-2867
25. Ron, D., Brasier, A.R., Wright, K.A., Tate, J.E., and Habener, J.F. (1990) Mol. Cell. Biol. 10, 1023-1032
26. Wilson, D.R., Juan, T.S.-C., Wilde, M.D., Fey, G.H., and Darlington, G.J. (1990) Mol. Cell. Biol. 10, 6181-6191
27. Li, X., and Liao, W. S.-L. (1991) J. Biol. Chem. 266, 15192-15201
28. Nishio, Y., Isshiki, H., Kishimoto, T. and Akira, S. (1993) Mol. Cell. Biol. 13, 1854-1862
29. Heinrich, P.C., Castell, J.V., and Andus, T. (1990) Biochem. J. 265, 621-636
30. Schooltink, H., Schmitz-Van de Leur H., Heinrich, P.C. and Rose-John S. (1992) FEBS Lett. 297, 263-265