AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1

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Received 14 April 1999; accepted in final form 31 January 2000

Lee, Patty J., Sharon L. Cambi, Beek Yoke Chin, Jawed Alam, and Augustine M. K. Choi. AP-1 and STAT mediate hyperoxia-induced gene transcription of heme oxygenase-1. Am J Physiol Lung Cell Mol Physiol 279: L175–L182, 2000.—We have previously shown marked induction of the stress-inducible gene heme oxygenase-1 (HO-1) in vivo and in vitro after hyperoxia. In RAW 264.7 cells, HO-1 induction is transcriptionally regulated and dependent on cooperation between the HO-1 gene promoter and the 5‘ distal enhancer element SX2. In our present study, further deletional and mutational analyses demonstrate that signal transducer and activator of transcription (STAT) DNA binding sites located in the promoter of HO-1 and activator protein (AP)-1 DNA binding sites in the distal enhancer element SX2 are necessary for optimal HO-1 gene activation after hyperoxia. Interestingly, a second 5‘ distal enhancer element, AB1, located 10 kb upstream from the HO-1 promoter, alone is activated after hyperoxia but cannot confer maximal hyperoxia-induced HO-1 gene transcription. Muta-
tional analysis of the AB1 enhancer shows that AP-1 is essential for AB1-mediated HO-1 gene transcription after hyperoxia. Electromobility shift assays show increased STAT1, STAT3, STAT5, and AP-1 DNA binding activity in RAW 264.7 cells after hyperoxia. Taken together, our data suggest that the 5‘ distal enhancer elements of the HO-1 gene in concert with the promoter regulate HO-1 gene induction and highlight the complexity of HO-1 gene transcription in response to hyperoxia.

signal transducer and activator of transcription; activator protein-1; gene regulation; oxidative stress; transcription factor; oxygen

AEROBIC ORGANISMS HAVE EVOLVED a variety of complex mechanisms to balance the need for oxygen to survive with the need to defend against potential oxygen toxicity. The classically described antioxidant defenses are the cellular enzymes such as manganese superoxide dismutase, copper-zinc superoxide dismutase, catalase, and glutathione peroxidase, which act to detoxify the mediators of oxidant stress, reactive oxygen species (7, 10, 45, 46). Recently, heme oxygenase (HO)-1, a ubiquitous eukaryotic enzyme, has generated much interest as a novel stress protein that is highly induced by oxidative stress including hyperoxia. HO catalyzes the initial and rate-limiting steps in the oxidative degradation of heme to bilirubin (39, 40). HO exists in three isoforms; HO-2 and HO-3 are primarily constitutive, whereas HO-1 is highly inducible (24, 25). Oxidants such as ultraviolet irradiation, sodium arsenite, heavy metals and, recently, hyperoxia have been found to be potent HO-1 inducers (5, 17–19, 22).

HO-1 induction in response to agents causing oxidative stress may reflect a protective strategy of the cell to defend against noxious stimuli including ultraviolet irradiation and hemoglobin toxicity in vitro (1, 43, 44). In vivo protective effects of HO-1 have also been demonstrated in rat models of oxidant-mediated rhabdomyolysis (27) and lipopolysaccharide (LPS)-induced sepsis (28). Our laboratory has also described that HO-1 overexpression in pulmonary epithelial cells conferred increased cell survival against lethal concentrations of hyperoxia in vitro (23) and that exogenous administration of HO-1 via a transgene approach provided protection against hyperoxia in vivo (29). A recent study by Poss and Tonegawa (31) demonstrating the increased susceptibility of HO-1-null knockout mice to oxidative stress further strengthens the emerging paradigm that HO-1 is indeed an important molecule in the host and cell defense against oxidant stress.

Given accumulating evidence for the important role of HO-1 in defense against oxidative stress, including...
hypoxia, and the fact that very little, if any, information exists regarding the molecular regulation of HO-1 gene expression in response to hypoxia, our laboratory sought to examine the molecular regulation of HO-1 gene expression after hyperoxic stress. Lee et al. (22) have previously observed that HO-1 expression is markedly induced by hypoxia in vivo and in vitro at the level of mRNA and protein and that increased HO-1 gene expression is transcriptionally regulated after hypoxia. Our current study attempts to better understand the transcriptional regulation of the HO-1 gene by identifying the cis-acting regulatory elements in the HO-1 promoter and 5’ distal enhancers that may regulate hypoxia-induced HO-1 gene transcription. We confirmed that both distal enhancers and the promoter of the HO-1 gene are important in mediating HO-1 gene transcription in response to hypoxia. Using deletional and mutational analyses of the HO-1 gene and electrophoretic mobility shift assays, we show that the transcription factors activator protein (AP)-1 and signal transducer and activator of transcription (STAT) both serve key roles in mediating HO-1 gene activation after hyperoxic stress.

METHODS

Tissue culture. Murine peritoneal macrophage cells (RAW 264.7) were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco’s modified Eagle’s medium (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Hy-Clone Laboratories, Logan, UT) and gentamicin (50 μg/ml). The cultures were maintained at 37°C in a humidified atmosphere of 95% air-5% CO2. The cells were exposed to hypoxia (95% O2-5% CO2) in a tightly sealed modular chamber (Billups-Rothenberg, Del Mar; CA) at 37°C. Because hyperoxia inhibits cell growth, all experiments were conducted in quiescent confluent cells in serum-free medium to avoid cell density variability between control and hypoxia-exposed cells during the course of the experiment.

Plasmids and transfections. The construction and characterization of pMHO1CAT, the mouse extended HO-1 promoter (1.3 kb) linked to the reporter gene chloramphenicol acetyltransferase (CAT) has been previously described (4). The construct pMHO4CAT contains 7 kb of the HO-1 5’-flanking region linked to the reporter gene CAT. The construct pMHO1CAT33+AB1 contains the HO-1 minimal promoter (33 bp) and a second 5’ distal enhancer site, AB1, located 10 kb from the transcription initiation site. Plasmids (10 μg) were stably cotransfected into RAW 264.7 cells with pcDNA 3-Neo (1 μg), a plasmid containing a neomycin selection marker, with LIPOFECTIN reagent (GIBCO BRL) according to manufacturer’s protocol. The cells were transfected for 24 h, after which time the plates were washed twice with serum-free medium and then incubated in DMEM containing 10% fetal bovine serum, 50 μg/ml of gentamicin, and 100 μg/ml of GENETICIN (GIBCO BRL). Approximately every 3 days, the concentration of GENETICIN in the medium was increased up to a maximum dose of 800 μg/ml. The surviving colonies (neomycin resistant) on each plate were pooled to establish the sublines.

Site-directed mutagenesis. The mutant plasmids pMHO1CAT+Sx2M2 (two AP-1 sites mutated) and pMHO1CAT+Sx2M4 (two CCAAT enhancer binding protein (C/EBP) sites mutated) have been previously described (2, 4).

Similar methods of oligonucleotide-directed mutagenesis were used to construct mutated AB1 sites and mutated STAT sites.

CAT assay. Celluar protein extracts were harvested after 24 h of hyperoxic exposure. The cells were washed with cold PBS before they were resuspended in 1.0 ml of 0.125 M Tris- HCl (pH 7.5) and then lysed by three cycles of freezing and thawing. Cell debris was then removed by centrifugation for 10 min at 14,000 g. Protein concentrations of the supernatants used for CAT assays were determined by Coomassie blue dye-binding assay (Bio-Rad Laboratories). In a reaction mixture of 150 μl of 20 mM acetyl-CoA and 0.3 μCi of [14C]chloramphenicol (Amersham), 100 μg of protein were incubated for 16 h at 37°C. Equal amounts of protein were used for each sample. The amount of acetylation was then determined by counting the acetylated and nonacyetylated forms that were separated by ascending thin-layer chromatography. The percentages of chloramphenicol acetylation were obtained over the linear range of the assay (30–50%) for each sample and were normalized for the protein content of the samples. If the assay gave >50% conversion of chloramphenicol, the assay was repeated for a shorter period of incubation time or with less cell extract. Mock transfections show a chloramphenicol conversion range of <0.5%. The SE was <20% for all values shown.

Cellular nuclear protein extraction. The cells were scraped in cold PBS and centrifuged at 14,000 g at 4°C for 10 min. After the supernatant was discarded, the cell pellet was lysed in buffer containing 10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol (DTT), 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was chilled in ice for 5 min and then centrifuged at 1,500 g to obtain cellular nuclei. The nuclei were washed in lysis buffer without Nonidet P-40 and centrifuged again at 1,500 g for 5 min. The supernatant was removed, and the pellet was resuspended in nuclear resuspension buffer containing 25 mM Tris, pH 7.8, 60 mM KCl, 1 mM DTT, and 1 mM PMSF. The nuclei were then frozen and thawed three times to obtain nuclear protein. The protein was kept in nuclear resuspension buffer and stored at –70°C. Protein concentrations used for electrophoretic mobility shift assay were determined by Coomassie blue dye-binding assay (Bio-Rad Laboratories).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were performed as previously described (6) with minor modifications. Briefly, DNA-binding activity was determined after incubation of 5.0 μg of RAW 264.7 cell nuclear protein extract with 20 fmol (20,000–50,000 counts/ min) of a 32P-labeled 25-mer oligonucleotide encompassing the generalized consensus STAT sequence (5’-GATCCCTAAGAGTTCGGGGAAGGTA-3’), the STAT1 sequence (5’-CATGTTATGCATATTCTGTTAAGTG-3’), the STAT3 sequence (5’-GATCCCTTCTGGGAAATTCCTAGATC-3’), or the AP-1 sequence (5’-AGATTTCTAGGAATTCAATCC-3’). After a 20-min incubation, the reaction mixture was electrophoresed on a 6% polyacrylamide gel (2% bis-acrylamide, 6.5% Tris-borate-EDTA, and 0.08% ammonium persulfate). The gel was transferred to DE81 ion-exchange chromatography paper (Whatman, Maidstone, UK) and dried down before exposure to autoradiographic film. Self-competitions were carried out under the same conditions with a 200-fold molar excess of the unlabeled STAT oligonucleotide probe. Nonspecific competitions were similarly performed with an unlabeled oligonucleotide probe.
encompassing a C/EBP transcription factor binding site (5'-GATCATTTCCTCACTGCTCATTTCCTCA-3'). STAT supershifts were performed with antibodies to STAT1, STAT2, STAT3, STAT5a, and STAT5b that were generously provided by Dr. Andrew Larner (US Food and Drug Administration, Bethesda, MD). AP-1 mobility shift assays and competitions were performed as previously described (22).

RESULTS

Deletional analysis of HO-1 gene and its 5' distal flanking sequences after hyperoxia. In our previous study, we determined HO-1 to be transcriptionally regulated after hyperoxia and dependent on cooperation between the 5' distal enhancer SX2 and the proximal promoter pMHO1CAT (22). In our previous study, transfection of RAW 264.7 cells with the full HO-1 gene construct, pMHO11CAT, yielded a 5.7 ± 0.76-fold induction after hyperoxia compared with that in cells in normoxia. Further deletions of the 5'-flanking region of the mouse HO-1 gene demonstrated that neither the HO-1 gene minimal promoter alone (pMHO1CAT) nor the fragment that includes the full promoter but not the SX2 enhancer (pMHO3CAT) is activated after hyperoxia (22). Surprisingly, pMHO1CAT331AB1 alone was also not responsive to hyperoxia (22) in contrast to earlier studies by our laboratory (2, 6, 32) showing that the SX2 enhancer alone is functionally active after various stimuli as endotoxin, heme, cadmium, and electrophiles. However, when the SX2 enhancer is linked to the full promoter (pMHO1CAT+SX2), full activation (5.4 ± 0.3-fold induction) of the HO-1 gene was observed after hyperoxia. Figure 1 demonstrates full HO-1 gene transcription (4 ± 0.23-fold induction) with the pMHO4CAT construct that encompasses the promoter and SX2 enhancer site. The difference in HO-1 induction between pMHO4CAT (4 ± 0.23-fold) and pMHO1CAT+SX2 (5.4 ± 0.3-fold) from a previous study (22) is not significant. Interestingly, hyperoxia did activate the second distal enhancer alone (pMHO1CAT331AB1). The 3.1 ± 0.23-fold induction with the pMHO1CAT331AB1 construct is significant compared with the HO-1 gene promoter alone (pMHO1CAT), albeit not to the extent observed with pMHO4CAT.

Deletional analysis of the HO-1 promoter pMHO1CAT after hyperoxia. Based on the above observation that full activation of the HO-1 gene requires both the promoter and the SX2 enhancer, we first performed sequential deletions of the HO-1 promoter (pMHO1) ranging from −44 to −2,1287 bp with an intact SX2 enhancer. Figure 2 demonstrates an 8.3-fold induction of CAT activity with the construct encompassing up to −2,592 bp, which statistically increased from the induction observed with −1,287 bp.
(5.4 ± 0.3-fold; Fig. 2). More importantly, however, the −592-bp construct alone is able to restore full HO-1 gene transcription, with a significant inhibition of CAT activity when the sequences between −319 and −592 bp were deleted. Of note, two STAT consensus binding sequences (TTCCGGGAA and TTCTGGAAA) exist in this region. The putative STAT sites in the HO-1 proximal promoter are consistent with the known γ-activated sequence (consensus, TTNNNNNAA, where N is any nucleotide) (16). As shown in Fig. 3, the presence of only a single STAT site reduced CAT activity 2.9-fold. When both STAT sites are mutated, however, CAT activity was completely abolished.

Mutational analysis of the SX2 enhancer after hyperoxia. Because full activation of the HO-1 gene requires both the promoter and the SX2 enhancer, we then generated additional stable cell transfectants possessing an intact full promoter but modifications of the SX2 enhancer site (Fig. 4). The SX2 fragment contains two putative AP-1 sites as well as two C/EBP binding sites (Fig. 4). Mutating the AP-1 sites (pMHO1CAT+SX2M2) significantly decreased CAT activity from 5.4- to 3.3-fold induction after hyperoxia but did not abolish the activity completely. Mutations of the C/EBP DNA binding site had no significant effect on hyperoxia-induced HO-1 gene transcription (Fig. 4).

The second distal enhancer AB1 alone can also activate HO-1 gene transcription after hyperoxia. A second putative 5’ distal enhancer site, AB1, located 10 kb upstream from the transcription initiation site, has recently been identified and has been implicated in the induction of the HO-1 gene transcription in response to other stimuli (3). We examined whether this second distal enhancer, AB1, alone also mediates hyperoxia-induced HO-1 gene transcription. Figure 5 shows a 3.1-fold induction of CAT activity by AB1. AB1, which alone was able to induce HO-1 gene transcription (Fig. 1), also contains putative AP-1 and C/EBP sites (3). Figure 5 shows that mutating one or more AP-1 sites will decrease or completely ablate CAT activity. However, although AB1 can induce HO-1 gene transcription, the magnitude of induction is not to the level achieved by either the full promoter construct pMHO11CAT or the proximal promoter in cooperation with SX2 (pMHO1CAT+SX2).

Transcription factors STAT and AP-1 are activated after hyperoxia. To further confirm the role of both STAT and AP-1 activation after hyperoxia, we performed electrophoretic mobility shift assays using a synthetic, double-stranded DNA probe specific for the consensus sequences for STAT1, STAT3, STAT5, and AP-1 binding sites, respectively. Figure 6A shows increased STAT binding activity in RAW 264.7 cells after 30 min of hyperoxic exposure, with a return to basal levels by 1 h for STAT1 and STAT3 and by 4 h for STAT5. The specificity of the STAT binding is demonstrated by the ability of unlabeled (cold) STAT to compete with the radiolabeled STAT for binding, whereas unlabeled C/EBP, an unrelated oligonucleotide, did not compete for STAT binding (Fig. 6B). To further confirm
the specific STAT proteins involved, mobility shift assays with antibodies to STAT1, STAT2, STAT3, STAT5a, and STAT5b were performed. Figure 6C shows new supershifted bands to STAT1, STAT2, STAT3, STAT5a, and STAT5b. Lee et al. (22) have previously demonstrated AP-1 binding activity in RAW 264.7 cells after 4 h of hyperoxic exposure. We extended the time course of AP-1 binding activity in Fig. 7A to include 30 min and 1 h of hyperoxia. AP-1 binding activity increases by 30 min of hyperoxic exposure and remains elevated by 4 h.

**DISCUSSION**

Hyperoxia is a well-established model of oxidant-mediated lung injury, with the presumed mechanism to be through reactive oxygen species. The lungs respond to the deleterious effects of reactive oxygen species such as superoxide and hydroxyl radicals and hydrogen peroxide by modulating levels of antioxidant enzymes and stress response proteins (9, 10). One such stress response protein after hyperoxic insult is HO-1, which Lee et al. (22) have previously shown to be highly upregulated at the level of gene transcription. Furthermore, hyperoxia-induced HO-1 gene transcription correlates with increased protein and activity levels after hyperoxia in vivo and in vitro (22). In contrast, a study by Clerch and Massaro (10) shows that mRNA induction of the antioxidant manganese superoxide dismutase after hyperoxia is not correlated with increased enzyme activity, whereas the antioxidants copper-zinc superoxide dismutase, catalase, and glutathione peroxidase have no change in mRNA levels with a decrease or no change in enzyme activity after hyperoxia. This differential regulation of various antioxidant enzymes points to the complex link between gene regulation and expression and underscores the need to explore the specific regulatory mechanisms involved in lung injury models. Of note, Dennerly et al. (14) showed that in neonatal rat lungs exposed to hyperoxia, steady-state HO-1 mRNA does not change significantly, whereas HO-1 activity was elevated, indicating that posttranscriptional mechanisms may be involved.

However, in our model, Lee et al. (22) have shown HO-1 protein and activity to be solely transcriptionally regulated, with significant HO-1 mRNA induction in vivo, in adult rat lungs, and in vitro that correlates with increased HO-1 protein and activity.

Little is known regarding the precise molecular mechanisms regulating HO-1 gene expression after hyperoxia. Our studies confirm that the transcriptional activation of the mouse HO-1 gene after hyperoxia is dependent on both the promoter pMHO1CAT and the 5′ distal enhancer SX2 because neither the promoter nor the SX2 enhancer alone is functionally activated by hyperoxia. This requirement for both the promoter and the distal enhancer for hyperoxia-induced HO-1 gene transcription is unique among all the inducers of HO-1 gene activation studied so far. For example, mouse HO-1 gene activation in response to agents such as heme, heavy metals, and LPS only requires the SX2 enhancer alone (2, 4, 6), whereas the promoter alone is not functionally active. Interestingly, the human HO-1 promoter (up to 4.5 kb) has recently been shown to be responsive to heme but not to hyperoxia or iron (15). The requirement of both the promoter and the SX2 enhancer for hyperoxia-induced murine HO-1 gene transcription implies a necessary cooperation between different regulatory elements within the promoter and the enhancer SX2. On further delineation of the specific cis-acting regulatory elements involved in HO-1 gene transcription after hyperoxia, the AP-1 sites of the SX2 enhancer in tandem with STAT binding sites in the promoter appear to be good candidates for this necessary cooperation for optimal gene HO-1 activation. Mutating the two STAT sites in pMHO1CAT while preserving the SX2 enhancer will completely ablate hyperoxia-induced HO-1 gene transcription (Fig. 3), and mutating the AP-1 sites in the SX2 enhancer with an intact proximal promoter will attenuate gene transcription from 5.4- to 3.3-fold induction (Fig. 4). Additionally, mutating one or more AP-1 sites in the AB1 distal enhancer will completely ablate gene transcription (Fig. 5).
Evidence for interactions and cooperation between transcription factors has been observed with transcriptional proteins such as AP-1, activating transcription factor-2, nuclear factor-κB, and the glucocorticoid receptor (33, 38, 41). There is evidence that STAT proteins will demonstrate cooperative DNA binding not only with other STAT family members but also with other proteins and transcription factors including the glucocorticoid receptor, c-Jun, c-myb, C/EBPα, and SP1 in vitro (11, 20, 35, 37, 42). Similar to our data that both AP-1 and STAT binding sites are necessary to confer maximal \( \text{HO-1} \) gene transcription in response to hyperoxia, Korzus et al. (20) reported that maximal induction of the human genes matrix metalloproteinase-1 and its inhibitor tissue inhibitor of metalloproteinase-1 by the cytokine oncostatin M requires synergy between AP-1 and STAT binding elements in vivo. David et al. (13) showed that extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) regulating STAT and AP-1, and, interestingly, our laboratory (30) has also found enhanced tyrosine phosphorylation of cellular proteins corresponding to ERK1/ERK2 after hyperoxia. These data further support the potential role of ERK/MAPK in regulating both AP-1 and STAT after hyperoxia.

The important role for STAT in \( \text{HO-1} \) gene activation after hyperoxia is intriguing given that the Janus kinase (JAK)-STAT pathway has been best described in the signal transduction and transcription of cytokines, growth factors, and hormones (12). More recently, reactive oxygen species have also been implicated in the activation of the JAK-STAT pathway (36). STATs reside in the cytoplasm or beneath the cell membrane in a latent, hypophosphorylated form. On ligand binding, a member(s) of the JAK tyrosine kinase family is activated, which then recruits STAT proteins and activates the STATs by tyrosine phosphorylation (12). The activated STATs form homo- or heterodimers and, with or without additional factors, migrate to the nucleus to initiate gene transcription (21, 47). Different STAT combinations interact differentially with related DNA response elements, thereby eliciting diverse yet specific responses to different ligands (47).

It is not clear at this juncture the precise transactivating factors (e.g., STAT family members) that regulate \( \text{HO-1} \) gene transcription after hyperoxia. Our studies suggest that STAT1, STAT3, and STAT5 are
involved after hyperoxia. There may be cooperation between more than one STAT protein, which has been previously described (47), as demonstrated by the activation of STAT1, STAT3, and STAT5 binding (Fig. 6A) and the ability of multiple STAT antibodies (to STAT1, STAT3, and STAT5) to supershift the protein-DNA complex after 30 min of hyperoxia (Fig. 6C). Cooperation between STAT1, STAT3, and STAT5 has been demonstrated in response to interleukin (IL)-2 family members (IL-2, IL-7, and IL-15) and epidermal growth factor (16). The tendency for STAT proteins to bind with other STAT members in heterodimeric or multimeric complexes may explain the double banding pattern observed in Fig. 6A, which has been observed by other investigators (20). In addition, STAT 3 has been found to migrate as a doublet, possibly due to incomplete renaturation in response to interferon (IFN)-γ (42). Furthermore, we cannot exclude the possibility that there may be other proteins that are not members of the STAT family present in the hyperoxia-induced complexes. This has been shown with IFN-α-activated complexes that include not only STAT1 and STAT2 but also a 48-kDa protein related to IFN-regulated factor and the myb families of DNA-binding proteins (42).

There has been recent evidence linking STAT proteins to cell growth arrest, differentiation, and mitogenesis (8, 26, 48). STAT1, for instance, has been found to mediate p21-induced cell growth arrest (8). The cell cycle “gatekeeper” p53 is also thought to regulate the JAK-STAT pathway through a novel proteasome (34). Given the previous observation by Lee et al. (23) that HO-1 appears to provide cellular protection against hyperoxic injury through growth arrest and our current study demonstrating active STAT sites on the HO-1 gene, it is tempting to speculate that STAT may be one mechanism involved in the growth arrest and cytoprotective properties of HO-1. Our data thus far offers further insight into the transcriptional regulation of the multifaceted HO-1 gene in response to hyperoxia. We are not aware of any previous reports of STAT activation in response to hyperoxia. Here we propose that hyperoxia will activate STAT and in the case of HO-1 induction requires cooperation with AP-1 elements in distal enhancers of the HO-1 gene to mediate hyperoxia-induced gene transcription.

P. J. Lee was supported by National Heart, Lung, and Blood Institute (NIHBI) Grant K08-HL-04034. A. M. K. Choi was supported by NHLBI Grants HL-55330 and HL-60234, an American Heart Association Established Investigator Award, and National Institute of Allergy and Infectious Diseases Grant AI-42365. J. Alam was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-43135.

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