Regulation of the Hypoxia-inducible Transcription Factor 1α by the Ubiquitin-Proteasome Pathway*

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Pekka J. Kallioš, William J. Wilson, Sallyann O’Brien, Yuichi Makino§, and Lorenz Poellinger¶
From the Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, S-171 77 Stockholm, Sweden

HIF-1α (hypoxia-inducible factor 1α) is a basic-helix-loop-helix PAS (Per/Arnt/Sim) transcription factor that, under hypoxic conditions, dimerizes with a partner factor, the basic-helix-loop-helix/PAS protein Arnt, to recognize hypoxia-responsive elements of target genes. It has recently been demonstrated that HIF-1α protein but not mRNA levels are dramatically up-regulated in response to hypoxia. Here we show that inhibitors of 26 S proteasome activity produced a dramatic accumulation of endogenous as well as transfected HIF-1α protein under normoxic conditions, whereas the levels of Arnt protein were not affected. HIF-1α was polyubiquitinated in vivo under normoxic conditions, indicating rapid degradation via the ubiquitin-proteasome pathway. This degradation process appeared to target a region within the C terminus of HIF-1α. Importantly, HIF-1α ubiquitination was drastically decreased under hypoxic conditions. Up-regulation of HIF-1α protein by proteasome inhibitors did not result in transcriptional activation of reporter genes, indicating either the requirement of additional regulatory steps to induce functional activity of HIF-1α or the inability of polyubiquitinated forms of HIF-1α to mediate hypoxic signal transduction. In support of both these notions, we demonstrate that HIF-1α showed hypoxia-dependent translocation from the cytoplasm to the nucleus and that this regulatory mechanism was severely impaired in the presence of proteasome inhibitors. Taken together, these data demonstrate that the mechanism of hypoxia-dependent activation of HIF-1α is a complex multistep process and that stabilization of HIF-1α protein levels is not sufficient to generate a functional form.

Hypoxia-inducible factor 1 (HIF-1) consists of a heterodimer of two basic helix-loop-helix PAS proteins, HIF-1α and Arnt. Upon decrease in oxygen tension, the activated HIF-1α-Arnt complex functions as a transcription factor to control the expression of genes encoding products aimed at restoring cellular homeostasis such as erythropoietin, vascular endothelial growth factor, and several glycolytic enzymes (reviewed in Ref. 1). Both HIF-1α and Arnt mRNAs are constitutively expressed in a number of mammalian cell lines under normoxic and hypoxic conditions, suggesting that functional activity of the HIF-1α-Arnt complex is regulated by some as yet unknown post-transcriptional mechanism(s). Recently we and others have shown that HIF-1α protein levels are rapidly and dramatically up-regulated upon exposure of target cells to hypoxia (2–4). However, the precise mechanism by which HIF-1α becomes activated during hypoxia remains unclear. This process may involve either increased translation or increased stability of the HIF-1α protein or both. It has previously been suggested that inhibition of protein synthesis by cycloheximide blocks induction of HIF-1α-Arnt DNA binding activity, indicating that the rate of synthesis of the HIF-1α subunit may have been altered (5, 6). In striking contrast to this report, the addition of cycloheximide after maximal hypoxic induction was reported to have no effect either on the protein levels of HIF-1α or on the DNA binding activity by the HIF-1α-Arnt complex (4), suggesting that an increase in protein stability rather than increased translation may primarily determine the induction response. Furthermore, it has been shown that the HIF-1α protein has a rapid turnover with an estimated half-life of 5 to 10 min upon return of target cells to normoxia after induction (7).

Recently, a basic helix-loop-helix PAS protein with high similarity to HIF-1α, EPAS1/HLF (endothelial PAS domain protein 1/HIF-1α-like factor), has been identified. After activation by hypoxia, this factor functions as a heterodimer with Arnt and can bind to the same DNA elements as the HIF-1α-Arnt complex (8–10). In contrast to the ubiquitous mRNA expression pattern of HIF-1α, expression of EPAS1/HLF mRNA is predominantly restricted to endothelial cells (9, 10).

For many short-lived eukaryotic proteins, conjugation to the short polypeptide ubiquitin is a mandatory step in their degradation. Highly selective degradation of target proteins is a central component of a great variety of cellular regulatory mechanisms, examples ranging from the progression of the cell cycle, maintenance of circadian rhythms, to the pathways controlling signal transduction and metabolism (11, 12). The advantages obtained by this regulatory mechanism are obvious. In addition to the irreversibility of the inactivation, the other major benefit is the speed by which new steady-state levels of regulatory molecules can be established.

Here we show that the HIF-1α protein is polyubiquitinated under normoxic conditions and that this ubiquitination activity is significantly decreased in response to hypoxic conditions. Moreover, HIF-1α levels were up-regulated under normoxic conditions in the presence of inhibitors of the proteasome, strongly suggesting that HIF-1α function is regulated by proteasome-mediated degradation. This degradation process appeared to target the C terminus of HIF-1α. Finally, we demonstrate that HIF-1α translocates from the cytoplasm to the nucleus in response to hypoxia and that this function as well as...
with or without 100 μM in phosphate-buffered saline containing 0.1% Triton X-100 for 9 h at
in a 80-cm² flask and, after transfection, split onto 60-mm dishes for expression levels of various constructs, cells were collectively transfected with control plasmid) were used per 60-mm dish. When comparing the expression of the transfected gene products, a major determinant of the protein levels is the turnover rate of the polypeptide (12, 15). Previously we and others have shown that hypoxic stimulation either by lowering the oxygen concentration or by exposing cells to hypoxia-mimicking (16) chemicals such as CoCl₂ or desferrioxamine leads to a rapid increase in cellular HIF-1α levels but not those of the partner factor Arnt (2, 4). Therefore we attempted to investigate whether blocking one of the major

the transactivation function of HIF-1α is perturbed in the presence of proteasome inhibitors.

EXPERIMENTAL PROCEDURES

Reagents—Proteasome inhibitors N-acetyl-Leu-Leu-norleucinal (LLeL) and clasto-lactacystin β-lactone were obtained from Sigma and Calbiochem, respectively. Expression plasmid encoding histidine-tagged ubiquitin (13) was a generous gift from Dr. Dirk Bohmann (EMBL, Heidelberg). The iron-chelating agent 2,2'-dipyridyl was purchased from Sigma. pFLAG CMV2- HIF-1α encoding FLAG epitope-tagged HIF-1α was assembled by subcloning in-frame the full-length coding sequence of HIF-1α from pGEX4T3- HIF-1α to BamHI/SmaI-digested pFLAG CMV2 (Eastman Kodak Co.). The construction of the various HIF-1α deletion mutants fused to the GAL4 DNA binding domain will be presented elsewhere.

Cell Culture and Transfections—COS7 cells (from ATCC) were routinely maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum plus penicillin (50 IU/ml) and streptomycin (50 μg/ml). The cells were transfected using Dotap (Boehringer Mannheim) according to the manufacturer’s recommendations. For ubiquitination assays, 6 μg of FLAG-tagged HIF-1α or the empty control plasmid in combination with 6 μg of His-tagged ubiquitin (or the control plasmid) were used per 60-mm dish. When comparing the expression levels of various constructs, cells were collectively transfected in a 80-cm² flask and, after transfection, split onto 60-mm dishes for treatments. Whole cell extracts were prepared as described previously (2). Primary mouse brain endothelial cells (kindly supplied by Dr. Yihai Cao, Karolinska Institute) were incubated in F12 medium containing 10% fetal calf serum and antibiotics. For transactivation experiments, cells were transiently transfected with pCMV-HIF-1α (14) together with a luciferase reporter gene carrying three tandem copies of the hypoxia response element of the erythropoietin gene (ERE-luc). Six h after transfection, cells received fresh medium and were either left nontreated or treated with 100 μM 2,2'-dipyridyl or 35 μM LLeL for 24 h before harvesting.

Immunostaining of HIF-1α—COS7 cells were grown on fibronectin-coated coverslips in 6-well culture plates and transiently transfected with pCMV-HIF-1α. After 24 h of expression, the cells were incubated in the presence or absence of 20 μM LLeL for 12 h and further incubated with or without 100 μM 2,2'-dipyridyl for 6 h. Thereafter, fixation of the cells was performed with a freshly prepared solution of 4% (v/v) paraformaldehyde in phosphate-buffered saline overnight at 4 °C. The fixed cells were incubated with anti-human HIF-1α rabbit antisera (2) in phosphate-buffered saline containing 0.1% Triton X-100 for 9 h at 4 °C. Indirect immunofluorescence was obtained by incubation with

biontylated goat anti-rabbit IgG antibodies and Texas red-conjugated streptavidin (Amersham Pharmacia Biotech) in phosphate-buffered saline containing 0.1% Triton X-100. The coverslips were mounted on glass slides and subjected to microscopic analysis.

Purification of Ubiquitin Conjugates—Twenty h after transfection, cells were washed with phosphate-buffered saline and lysed in 1.3 ml of 6 M guanidinium-HCl in 20 mM Heps, pH 7.9, per 60-mm dish. The lysate was passed through a 27-gauge needle to decrease viscosity, and the protein concentration was measured using a Bio-Rad protein assay. Twenty μl of Talon metal affinity resin (CLONTECH) was then added/ 900 μg of total protein. The lysate was rotated overnight at 4 °C in the presence of 10 mM imidazole. The matrix was then washed with decreasing concentrations of guanidinium-HCl buffers containing 1:2 and 1:4 dilutions of the original guanidinium-HCl buffer A (40 mM Heps, pH 7.9, 100 mM KCl, 15% glycerol) supplemented with 10 mM imidazole) and then finally washed with a 1:8 dilution of this buffer before washing with buffer A only. Remaining proteins were eluted with SDS sample buffer supplemented with 100 mM EDTA and analyzed by immunoblotting.

Immunoblotting and Detection—The detailed protocol for the immunoblotting of HIF-1α and Arnt proteins together with description of the rabbit antisera has been described previously (2). The anti-HIF-1α antisemur used is specific for HIF-1α, because it does not recognize in vitro translated EPAS/HLF (data not shown). For the detection of the FLAG epitope-tagged constructs or GAL4 fusion proteins, proteins were blotted after SDS-polyacrylamide electrophoresis onto nitrocellulose filters and blocked overnight with 5% nonfat milk in Tris-buffered saline. Either the anti-FLAG M2 antibody (Kodak) or anti-GAL4 antiserum (Upstate Biotechnology, Lake Placid, NY) were used as a primary antibody in a dilution of 10 μg of protein/ml of Tris-buffered saline containing 1% nonfat milk for 2 h. After several washes, a 1:750 dilution of anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech) in Tris-buffered saline, 1% nonfat milk was used as a secondary antibody. After extensive washing with Tris-buffered saline, the complexes were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

RESULTS

Inhibition of 26 S Proteasomal Activity Leads to Accumulation of Endogenous and Transiently Expressed HIF-1α Levels—In the case of a broad number of either inducible or periodically expressed gene products, a major determinant of the protein levels is the turnover rate of the polypeptide (12, 15). Previously we and others have shown that hypoxic stimulation either by lowering the oxygen concentration or by exposing cells to hypoxia-mimicking (16) chemicals such as CoCl₂ or desferrioxamine leads to a rapid increase in cellular HIF-1α levels but not those of the partner factor Arnt (2, 4). Therefore we attempted to investigate whether blocking one of the major
cellular protein degradation pathways, i.e., the pathway mediated by the 26 S proteasome, would lead to accumulation of HIF-1α protein also under normoxic conditions. To this end we exposed mouse brain primary endothelial cells to the peptide aldehyde LLnL, an inhibitor of the proteasomal pathway, and analyzed whole cell extracts by immunoblotting using HIF-1α-specific antisera. As shown in Fig. 1, treatment of mouse brain endothelial cells with 35 μM LLnL for 20 h resulted in a marked increase of endogenous HIF-1α protein levels (whereas most of the nonspecific cross-reacting signals remained unaffected). Because LLnL is also known to have an inhibitory effect on some nonproteasomal degradation pathways, e.g., those mediated by lysosomes or by calpains and cathepsins (17), we decided to perform the same experiment using clasto-lactacystin β-lactone, a highly selective inhibitor of 26 S proteasome activity (18). Lactacystin and its more potent derivative, clasto-lactacystin β-lactone, have both been shown to modify and thereby inactivate the catalytic β-subunits of the proteasome (17). Interestingly, incubation of the brain endothelial cells in the presence of 5 μM clasto-lactacystin β-lactone for 20 h also lead to a marked stabilization of HIF-1α protein levels as compared with cells treated with vehicle only (Fig. 1A, compare lanes 1 and 2). Furthermore, identical results were obtained in the mouse hepatoma cell line Hepa 1c1c7 (data not shown). In parallel experiments, we examined the effect of the two proteasome inhibitors, clasto-lactacystin β-lactone and LLnL, on intracellular levels of Arnt in primary mouse brain endothelial cells. In contrast to HIF-1α protein levels, Arnt protein levels remained unaffected upon exposure to these inhibitors (Fig. 1B), in excellent agreement with the observation that hypoxic treatment does not alter Arnt protein levels (2). Taken together, these results clearly demonstrate that HIF-1α protein levels are specifically up-regulated in the presence of proteasome inhibitors and that this enhanced accumulation of protein is associated with increased metabolic stability.

To examine whether transiently expressed HIF-1α follows the same mode of regulation as the endogenous protein, we transiently transfected COS7 cells with a FLAG epitope-tagged HIF-1α expression vector and, 20 h after transfection, exposed the cells to either inducers of the hypoxic response or to proteasome inhibitors. These conditions were identical to those used above when studying endogenous HIF-1α protein levels. After induction for 24 h, the cells were harvested and analyzed by Western blotting for the presence of the transfected protein. As seen in Fig. 2, even the high level of expression usually obtained after transfection into COS7 cells did not allow us to detect by immunoblot analysis any significant levels of FLAG-HIF-1α expression in normoxic cells, suggesting also that...
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Transfected HIF-1α is rapidly degraded. However, in analogy to the endogenous HIF-1α protein levels (2), exposure of cells to the hypoxia-mimicking iron chelator 2,2’-dipyridyl leads to a very significant accumulation of epitope-tagged HIF-1α. Thus, these data suggest that the increase in HIF-1α protein levels is mediated by sequences within the coding region of the gene product. Furthermore, in analogy to the endogenous HIF-1α, exposure of the transfected cells to proteasome inhibitors, either the peptide aldehyde LLnL or clasto-lactacystin β-lactone, dramatically increased the levels of epitope-tagged HIF-1α (Fig. 2; compare lanes 1, 3, and 4), indicating that the transiently expressed protein is rapidly degraded via the 26 S proteasome.

Up-regulation of HIF-1α Protein Levels by Proteasome Inhibitors Does Not Generate a Functional Form of HIF-1α—Under hypoxic conditions, up-regulation of HIF-1α protein levels correlates with induction of hypoxia-regulated genes and activation of reporter genes (1, 2). Given the up-regulation of both endogenous and transiently expressed HIF-1α protein levels upon exposure of cells to proteasome inhibitors under normoxic conditions, we next wanted to examine the effects of these inhibitors on functional activities of HIF-1α. In control experiments, treatment of mouse brain endothelial cells with 2,2’-dipyridyl resulted in potent activation of a hypoxia-responsive reporter gene construct (Fig. 3). Strikingly, however, exposure of the cells to increasing concentrations of the proteasome inhibitor LLnL under normoxic conditions did not result in activation of the reporter gene (Fig. 3). Thus, these results indicate that mere up-regulation of HIF-1α protein levels by proteasome inhibitors was not sufficient to elicit a transcriptional response. In conclusion, under these conditions, HIF-1α may not be functional, and/or additional regulatory steps may be important to activate HIF-1α function. In support of this model, 2,2’-dipyridyl-induced transcriptional activation by HIF-1α was inhibited in a dose-dependent manner by cotreatment of the cells with LLnL (Fig. 3).

Hypoxia-induced Nuclear Translocation of HIF-1α—We next transiently expressed HIF-1α in COS7 cells using a CMV pro-
motor-driven expression vector and studied the intracellular localization of HIF-1α. Under normoxic conditions, HIF-1α immunoreactivity of rather low intensity was preferentially localized in the cytoplasmic compartment of the cell, as assessed by immunostaining using polyclonal anti-HIF-1α antibodies. In contrast, exposure of the cells to the hypoxia-mimicking agent 2,2′-dipyridyl resulted in an almost complete nuclear translocation of HIF-1α with no detectable immunoreactivity remaining in the cytoplasm (Fig. 4). After incubation of the cells with the proteasome inhibitor LLnL under normoxic conditions, high intensity HIF-1α immunoreactivity was uniformly distributed throughout the cell. Strikingly, this intracellular distribution was not altered upon exposure of the cells to a combination of both LLnL and 2,2′-dipyridyl (Fig. 4). Thus, hypoxia-dependent nuclear accumulation of HIF-1α was impaired upon stabilization of HIF-1α protein levels by LLnL.

Increase in Protein Levels in Response to Hypoxia Is Mediated by Structures within the HIF-1α Protein—In our initial attempts to map the putative destabilizing elements within the HIF-1α protein, we transiently expressed in COS7 cells a set of HIF-1α deletion mutants fused to the minimal DNA binding domain of the yeast transcription factor GAL4 (schematically represented in Fig. 5A). In excellent agreement with the corresponding effects on endogenous HIF-1α levels (Fig. 1), GAL4-HIF-1α levels were significantly increased upon exposure of the cells to either 2,2′-dipyridyl, LLnL, or a combination of these two agents (Fig. 5B; compare lane 1 to lanes 2–4). A very similar mode of regulation of the protein levels was observed using the HIF-1α deletion mutant GAL4-HIF-1α 1–652. Importantly, the protein levels of GAL4-HIF-1α 526–826 spanning the C terminus of HIF-1α were also stabilized by treatment with either 2,2′-dipyridyl, LLnL, or a combination of both. Interestingly, the C terminus of HIF-1α (residues 526–826) harbors both an N-terminal and a C-terminal transactivation domain, which both act in concert with one another to mediate hypoxia-inducible transcriptional activation (19, 20). In addition, as outlined in Fig. 5A, this region of HIF-1α contains in the vicinity of the N-terminal transactivation domain a sequence with strong similarity to a PEST motif, which often targets proteins for rapid degradation (21). Apart from this motif, the C terminus of HIF-1α does not display any significant similarity to previously described protein-destabilizing elements. The C-terminal transactivation domain of HIF-1α contained in GAL4-HIF-1α 786–826 showed significant and stable steady-state levels of expression upon transient expression in normoxic COS7 cells. These stable protein levels were not further increased in response to hypoxic stimulation and only slightly elevated in the presence of proteasome inhibitors (Fig. 5B; compare lanes 13–16). In conclusion, these data indicate that the region spanning the N-terminal transactivation domain of HIF-1α also contains a structural motif involved in protein destabilization under normoxic conditions and, thus, shows a complex functional architecture. It is noteworthy that EPAS/HLF, which also mediates hypoxia-inducible transcriptional activation (9, 10), contains a PEST motif in a very similar region of the protein. Consistent with this observation and in analogy to GAL4 HIF-1α, GAL4 HIF-1α 1–652, and GAL4 HIF-1α 526–826, GAL4 HLF steady-state protein levels were hardly detectable upon transient expression in COS7 cells under normoxic conditions and significantly stabilized after exposure to hypoxic stimulation, proteasome inhibitor, or both treatments (data not shown). In contrast, in control experiments, the minimal GAL4 DNA binding domain was stably expressed under normoxic conditions and showed only modest stabilization in the presence of proteasome inhibitor (Fig. 5C).

Degradation of HIF-1α via the Ubiquitin-Proteasome Pathway—To directly assess whether HIF-1α can form ubiquitin conjugates in vivo, COS7 cells were cotransfected with an HIF-1α expression vector together with a vector expressing multimerized polyhistidine-tagged ubiquitin (H₆-ub, lanes 2, 3, and 5–7) or with corresponding control vectors (lanes 1 and 4). After transfection, cells were treated for 20 h with 35 μM LLnL (A), 10 μM clasto-lactacystin β-lactone (B) (lanes 1, 3, 4, 6, and 7), or vehicle (Me₂SO) only (lanes 2 and 5). His-tagged proteins were purified from the transfected cell lysates using a metal-affinity column, and specifically bound material was then analyzed by immunoblotting using anti-FLAG (A) and anti-HIF-1α antiserum (B). The sample in lane 7 was treated identically as that in lane 6 except during the 20-h induction period with the chemicals, lane 7 was simultaneously exposed to 100 μM 2,2′-dipyridyl (DP), mimicking hypoxic induction. For each panel, the positions of the molecular weight markers are shown on the left.
FIG. 7. Role of ubiquitin (Ubq)-mediated proteasomal degradation in activation of HIF-1α function. HIF-1α is multiubiquitinated under normoxic conditions and, following ubiquitination, rapidly degraded via the 26 S proteasome. Hypoxic stimulation allows HIF-1α to escape ubiquitination, leading to an increase in protein levels, induced nuclear import, and dimerization with Arnt, enabling HIF-1α to recognize cognate hypoxia response elements and thereby activate target gene transcription.

Activity are specifically because of increased levels of HIF-1α protein, because Arnt levels were unaltered in the presence of proteasome inhibitors. Although the DNA binding activity of the HIF-1α-Arnt complex appears to be induced under conditions of proteasome inhibition (25), this response did not correlate with activation of hypoxia response element-dependent reporter gene expression, suggesting that a nonfunctional form of HIF-1α is accumulated. Moreover, in excellent agreement with the lack of functional activity of HIF-1α upon stabilization by proteasome inhibition, our data indicate the failure of HIF-1α to accumulate in the nucleus of hypoxic cells in the presence of proteasome inhibitors. Taken together, these data strongly argue that polyubiquitinated forms of HIF-1α that are generated in the presence of proteasome inhibitors are nonfunctional.

To address the possible involvement of polyubiquitination in targeting HIF-1α for destruction, we used an in vivo assay (13) where, upon transient expression of affinity-tagged ubiquitin, target proteins can be isolated using an affinity resin. Employing this assay in the presence of a proteasome inhibitor to prevent rapid destruction of the conjugated complexes, HIF-1α was effectively copurified in the presence of histidine-tagged ubiquitin. This effect was selective for HIF-1α because no immunoreactivity was detected in the absence of either His-tagged ubiquitin or proteasome inhibitors. Furthermore, the finding that HIF-1α is multiubiquitinated under normoxia may be of potential importance, because it has been demonstrated that not all proteins targeted to the 26 S proteasome are ubiquitinated; it has rather been shown that proteasome may have a more general function in selective removal of short-lived proteins by recognizing degradation signals other than ubiquitin (26).

Interestingly, exposure of cells to an hypoxia-mimicking agent simultaneously with the proteasome inhibitors resulted in marked reduction of ubiquitin-bound HIF-1α, which correlated with elevated cellular protein levels. Thus, as outlined in the model in Fig. 7, HIF-1α is differentially regulated by ubiquitination in normoxia as opposed to hypoxia, and inhibition of ubiquitination constitutes an early and critical step in regulation of HIF-1α function preceding nuclear translocation, recruitment of Arnt, and ensuing gene activation. Our present experiments indicate that regulation of HIF-1α protein levels by the proteasome pathway were mediated by a C-terminal structure of the protein spanning a PEST sequence motif (21). Interestingly, the same region of HIF-1α has been shown to harbor the N-terminal transactivation domain of HIF-1α and
has thus been implicated to be involved in conditional regulation of HIF-1α function (19, 20). Thus, these data illustrate the very complex functional architecture of the C terminus of HIF-1α. In agreement with the present results, the identification of a broadly defined oxygen-dependent degradation domain between residues 401 and 603 was reported during the completion of this study (27). What is the degradation motif (or motifs) within this C-terminal region of HIF-1α and what is the nature of the signal and mechanism that renders the degradation process inactive during hypoxic conditions? Transplantable sequence elements, destruction boxes, recognized and targeted by a proteolytic apparatus have been identified in many short-lived proteins (28), but despite this, the structural features characterizing these elements are largely unknown. Against this background, HIF-1α provides an interesting model system to understand regulation of protein function by protein degradation. In particular, it will be important to investigate how the process of HIF-1α protein stabilization is related to the mechanism of conditional regulation of HIF-1α function.

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