Amino Acid Analogs Activate NF-κB through Redox-dependent IκB-α Degradation by the Proteasome without Apparent IκB-α Phosphorylation

CONSEQUENCE ON HIV-1 LONG TERMINAL REPEAT ACTIVATION*

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We report here that amino acid analogs, which activate hsp70 promoter, are powerful transcriptional activators of human immunodeficiency virus 1 (HIV-1) long terminal repeat (LTR), an activation which was impaired when the two kB sites present in the LTR were mutated or deleted. Amino acid analogs also stimulated the transcription of a kB-controlled reporter gene. Upon treatment with amino acid analogs, the two NF-kB subunits (p65 and p50), which are characterized by a relatively long half-life, redistributed into the nucleus where they bound to kB elements. This phenomenon, which began to be detectable after 1 h of treatment, was concomitant with the degradation of the short lived inhibitory subunit IκB-α by the proteasome. However, contrasting with other NF-kB inducers that trigger IκB-α degradation through a phosphorylation step, amino acid analogs did not change IκB-α isoform composition. Antioxidant conditions inhibited amino acid analog stimulatory action toward NF-kB. This suggests that aberrant protein conformation probably generates a pro-oxidant state that is necessary for IκB-α proteolysis by the proteasome. Moreover, this activation of NF-kB appeared different from that mediated by endoplasmic reticulum overload as it was not inhibited by calcium chelation.

HIV-1 infection is characterized by a phase of clinical latency in the course of which the rate of replication of the virus is important, notably in lymphoid organs (1, 2). Other cells that are infected in a latent manner necessitate a reactivation to generate viral production. Stimuli that mediate such reactivation include cytokines, phorbol esters, tumor promoters, protein kinase inhibitors (3), co-infection by other viruses (4, 5), cadmium, arsenite (6), oxidative conditions (7), or thermal stress (8–10). These stimuli regulate the transcription of the HIV-1 provirus through the modulation of the complex eukaryotic promoter localized in the long terminal repeat (LTR). This promoter contains binding sites for many transcription factors, including NF-kB, SP1, USF, and AP1 (11, 12). In the case of oxidative stress mediated by either hydrogen peroxide or tumor necrosis factor-α (TNF-α), the transcriptional activation of HIV-1 LTR was clearly shown to depend on NF-kB (7). In contrast, HIV-1 LTR activation by protein kinase inhibitors has not yet been demonstrated to require any specific transcription factor (3). Similarly, despite striking similarities between the kinetics of thermal activation of the LTR and the heat shock promoter (HSE), the molecular mechanism leading to the activation of HIV-1 LTR by heat shock is still not understood (9, 10).

Heat shock, which induces the accumulation of misfolded or damaged proteins, results in the preferential expression of heat shock or stress proteins (hsp), which contribute in helping the cell to recover from thermal damage (13–18). Other conditions or agents that interfere with protein folding as well as denatured proteins are usually stress protein inducers. They include denatured bovine serum albumin, mutated actin, heavy metals, arsenite, and amino acid analogs (18–21). The latter agents are structural analogs of natural amino acids and are therefore rapidly incorporated into newly synthesized proteins (19, 22). Hence, they induce irreversible aberrant protein conformations that lead to the induction of stress proteins, such as hsp70, and glucose-regulated proteins, i.e. Grp78/Bip (17, 23–25).

NF-kB belongs to a family of inducible transcription factors, the Rel/NF-kB family (26–28). In addition to being a major enhancer of the HIV-1 LTR promoter, NF-kB regulates the expression of numerous cellular genes, particularly those involved in the immune and inflammatory responses (29–32). NF-kB activation is under the control of the IκB family of inhibitory subunits. The most studied IκB protein is IκB-α which associates in the cytoplasm with NF-kB dimer (33–36). This interaction masks the nuclear localization signal of NF-kB (37, 38) and therefore inhibits the nuclear translocation of this dimeric factor. NF-kB is activated by a large number of signals, which include inflammatory cytokines, phorbol esters (phorbol 12-myristate 13-acetate) (39), pathogenic agents (32), UV irra-
of oxidant drugs (7) or by the overexpression of detoxificant enzymes such as catalase (51) or glutathione peroxidase (52), suggesting that crucial redox events or intracellular reactive oxygen species are involved in NF-κB activation. Recently, an alternative mechanism of NF-κB activation has been reported to occur in Jurkat T cells stimulated with peroxovanadate. In this case, tyrosine phosphorylation of IκB-α activates NF-κB without proteolytic degradation of IκB-α (53). However, redox modulation of this phenomenon, (ROS are potent inhibitors of tyrosine phosphatases (54)) is still a matter of debate (53, 55, 56).

Independently of its rapid breakdown induced by several stimuli and compared with NF-κB protein subunits, IκB-α is inherently unstable and undergoes a continuous turnover. This phenomenon does not require the prior phosphorylation or ubiquitination of IκB-α but still appears mediated by the proteasome (57).

Since HIV-1 LTR is transcriptionally activated by several cellular stimuli, including heat shock (6, 8–10), we tested whether the accumulation of aberrantly conformed proteins could trigger the activity of this promoter. To this end, the inducible activity of amino acid analogs have been tested. We demonstrate here that azetidine or canavanine, which mimic proline and arginine, respectively, are powerful transcriptional activators of HIV-1 LTR. This stimulation, which necessitated the κB sites present in the LTR, was impaired by antioxidants in contrast. hsp70 promoter activation by amino acid analogs was stimulated by antioxidant conditions. Concomitantly with HIV-1 LTR activation, we show that amino acid analogs induce the proteasome-mediated degradation of the inhibitory subunit IκB-α without a prior change in its isoform distribution. Hence, aberrant protein conformation appears to generate pro-oxidant conditions that trigger the degradation of short lived and probably misfolded proteins, such as IκB-α, and subsequently the activation of NF-κB, a phenomenon that results in HIV-1 transcriptional stimulation.

MATERIALS AND METHODS

Cell Cultures—HeLa cells were grown at 37 °C in the presence of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (Life Technologies, Inc., France). 0.1 mg fresh sodium selenite, 2 mM L-glutamine, 0.5 μg/ml insulin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Prior to amino acid analog treatments, cells were incubated 30 min in DMEM or RPMI medium supplemented with 5 or 10% dialyzed fetal calf serum. The proteasome inhibitor N-β-(3-2-bromo-3-carboxyloxy-4-nitrophenyl)histidine (BAPTA-AM) was from Calbiochem (Meudon, France). Anti-p65RelA, anti-p50, and anti-IκB-α/MAD-3 were from Santa Cruz Biotechnology (Santa Cruz, CA). pLTR-cat, p17-cat-neo, and pSV2-cat plasmids were described elsewhere (10). pB2X-3’TTKcat vector, which is made of two artificially constructed κB sites in front of a cat gene, has already been described (60). pCMVβ is a plasmid from CLONTECH (Palo Alto, CA) that contains the β-galactosidase gene under the control of the cytomegalovirus promoter. pLTR-Psi1-GOCCTTAAGCACGAG (Promega, France) contains that the first two bases of the two consensus κB sequences present in the LTR were mutated. This mutant was produced by megaprimer polymerase chain reaction mutagenesis (61). Primer for the entire HIV-1 LTR were upstream primer (5’-GGGCTCGAGAAGCTTCAGGAAACATTTG-3’) and downstream (reverse) primer (5’-CGGCAAGCTTAGGTTTTATAATT-3’), containing appropriate sites for XhoI and HindIII. The mutagenic primer (5’-TTGCTAACAGACTTCCGCTGACGTTTTCGAG-3’) created a Psi1 site between the two mutated κB κB consensus binding sites. Mutated bases are in boldface type. A first round of amplification with the mutagenic and downstream primers was used to amplify the 3’ region of the HIV-1 LTR. The resulting fragment which was mutated in both NF-κB κB sites was gel purified and used as downstream primer in a subsequent reaction to amplify the entire HIV-1 LTR. The final polymerase chain reaction product of 0.7 kilobase pair was gel purified and the presence of the Psi1 site verified by digestion. The fragment was then digested with XhoI-XbaI and ligated into the pLTR-cat vector from which the wild type HIV-1 LTR had been removed by XhoI-XbaI digestion. Mutant plasmids which contained the full-length sequence with Psi1 and HindIII sites were sequenced to confirm the mutated sites and the absence of other mutations.

Transfection, CAT, and β-Galactosidase Assays—HeLa cells were seeded out 12 h before transfection at a density of 3 × 10⁶ cells/100-mm dishes. Transfection experiments were performed as described by Kretz-Remy and Ariggo (10). Briefly, 8 μg of the desired cat-containing plasmid described above were added to a transfection buffer containing 24 μl of Transfectam reagent (Promega, France) and then mixed with the 2-ml culture medium. Ten hours after transfection, cells were trypsinized and replated into four to six 60-mm dishes. Twelve hours later, cells were submitted to various amino acid analog treatments and were allowed to recover for 24 h prior to harvesting. Transfected cells were lysed, and 50 μg of total cellular proteins were analyzed using the Boehringer CAT-ELISA test according to the manufacturer’s instructions. The percentage of cells expressing β-galactosidase was monitored by 5-bromo-chloro-3-indolyl β-galactosidase staining (62).

Indirect Immunofluorescence Analysis—HeLa cells growing on glass coverslips coated with 0.1% of Type B gelatin were submitted to various treatments with amino acid analogs. They were then rinsed with PBS at 4 °C and fixed for 90 s with cold methanol. The coverslips were air dried and 1-100 in PBS containing 0.1% bovine serum albumin. Goat anti-rabbit immunoglobulin coupled to isothiocyanate was used as a second antibody (Organon Teknica-Cappel, Fresnes, France). The stained cells were examined and photographed with a Zeiss Axioskop photomicroscope. Fluorescent images were recorded onto Tri-X Pan (Eastman Kodak Co.) film.

Electrophoretic Mobility Shift Assays—Extraction of DNA-binding proteins and binding conditions have been previously described (10). Briefly, 10 μg of protein from nuclear extracts were incubated with a 20,000 cpm (Cerenkov) 3²P-labeled κB DNA probe in the presence of 4 μg of poly(dI-dC) (Pharmacia) and 1 μl of 10 × BB buffer. Reaction was for 15 min at room temperature following the addition of the 3²P-labeled κB probe. The double-strand oligonucleotide used to detect the DNA binding activity of NF-κB was as described previously (52, 63). For the competition experiments, 10 or 40 ng of cold κB probe were added to the binding mix including proteins just before the incubation with the 3²P-labeled κB probe. Supershift experiments were also performed by adding, 30 min before the incubation with the 3²P-labeled κB probe, 2 μg of an antibody raised against the p65/RelA subunit of NF-κB to the binding mix including proteins. Native 4% acrylamide gels were used to analyze the samples. Autoradiographs of the gels were recorded onto BioMax MR films (Eastman Kodak Co.).

Radiolabeling and Cellular Extraction—HeLa cells were pulse labeled with [³²]Smethionine and -cysteine (Trans²S²-label, 1500 Ci/mmol, ICN, Costa Mesa, CA) for different periods of time in methionine- and cysteine-free DMEM (ICN) containing 5% dialyzed fetal calf serum. Thereafter, cells were rinsed with PBS and scrapped off the dish. Before cellular extraction, aliquots were withdrawn for determination of protein concentration. For cellular extraction, 1.5 × 10⁶ HeLa or T47D cells grown in 60-mm cell culture dishes and submitted to various amino acid analog treatments were washed with cold PBS. The cells were then scraped from the dishes and pelleted for 5 min at 1000 × g. The pellet was then lysed and boiled in Laemmli sample buffer for 5 min.

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Gel Electrophoresis, Immunoblotting, and Immunoprecipitation—One- and two-dimensional gel electrophoresis and immunoblots were performed as already described using gels that contained 10% (45, 47, 69–71) or 12.5–15% (52) acrylamide. The isoelectrofocusing gels were made up with 60% of pH 4–6 and 40% of pH 3–10 Ampholines (Sigma Chimie, Saint-Quentin Fallavier, France). Isolelectrofocusing sample buffer contained pH 6–8 Ampholine. Immunoblots were probed with p65/RelA, p50, or IκB-α/MAD-3 antisera as primary antibodies and were revealed with the ECL kit from Amersham Corp. (United Kingdom). The duration of exposure was calculated to be in the linear response of the film. For immunoprecipitation under native conditions, a constant number of radiolabeled cells was analyzed. [35S]Methionine-labeled cells were rinsed in PBS, lysed in radioimmune precipitation buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM sodium deoxycholate, 0.1% SDS) and clarified for 10 min at 10,000 × g. Immunoprecipitation was performed by incubating cell lysates with anti-IκB-α or preimmune serum for 3 h on ice before incubation of 1 h in the presence of protein A-Sepharose CL4B (Sigma Chimie, Saint-Quentin Fallavier, France). Thereafter, the protein A-immunocomplexes were centrifuged for 15 s at 10,000 × g, washed several times with radioimmune precipitation buffer, and boiled in SDS sample buffer. After removal of protein A-Sepharose by centrifugation, samples were analyzed by SDS-PAGE and autoradiography.

RESULTS

Amino Acid Analog Activation of HIV-1 LTR—The transcriptional activity of HIV-1 LTR was tested in HeLa cells transiently transfected with pLTR-cat, a plasmid that contains the cat gene under the control of the HIV-1 LTR (see “Materials and Methods”). Parallel experiments were also performed with p17-cat-neo plasmid that contains the cat gene under the control of the promoter of the human hsp70 gene. Transfection efficiency at about 60%, tested by using the β-galactosidase gene-bearing pCMVβ plasmid, was similar in all experiments (not shown). Fig. 1A shows the pattern of activation of pLTR-cat and p17-cat-neo plasmids in HeLa cells exposed for 6 h to increasing concentrations of the proline analog, azetidine. The level of CAT polypeptide was quantified after a 24-h recovery period in a medium devoid of azetidine by CAT-ELISA testing (see “Materials and Methods”). A gradual increase of CAT polypeptide driven by pLTR-cat or p17-cat-neo plasmids was observed following treatment with increasing doses of azetidine. Of interest, the intensity of LTR activation was about 4-fold that of the hsp70 promoter. The reverse was observed when cells were exposed to heat shock (Fig. 1A). In addition, following exposure to 15 mM azetidine for different time periods, the kinetics of activation of HIV-1 LTR peaked after 6 h of treatment (Fig. 1B). As seen in Fig. 1, C and D, similar kinetics of activation of HIV-1 LTR and hsp70 promoter were also observed when canavanine, an arginine analog, was used instead of azetidine. In this case, however, the difference in the inducibility of the LTR versus hsp70 promoter was not as pronounced as in the case of azetidine.
promoter was only 2.5-fold instead of the 4-fold induced by azetidine. Control experiments were performed using the pSV2-cat plasmid that contains the cat gene under the control of the constitutive early promoter of SV40 virus. In this case, no change in the level of CAT polypeptide was induced by azetidine, canavanine (Fig. 1, E and F) or heat (not shown) (10). Hence, amino acid analogs act as potent activators of HIV-1 LTR.

We next investigated whether the amino acid analog activation of HIV-1 LTR resulted in transcriptional events. This was assessed by analyzing the effect of actinomycin D (0.5 μg/ml) on the production of the CAT polypeptide driven by HIV-1 LTR. To this end, transfected HeLa cells were treated for 6 h with increasing concentrations of either azetidine (Fig. 2A) or canavanine (Fig. 2B) and then allowed to recover for 3 h in a medium devoid of amino acid analogs. Actinomycin D was added 10 min prior to the beginning of the treatment with the analogs and the recovery period was of only 3 h to minimize the cytotoxic effect induced by actinomycin D. As seen in Fig. 2, A and B, actinomycin D abolished CAT polypeptide production induced by azetidine or canavanine, as compared with the level of this protein measured in cells not treated with actinomycin D (Fig. 2, A and B, plot C). Taken together, these results suggest that the activation of HIV-1 LTR by amino acid analogs is specific and transcriptionally regulated.

Transcriptional Activation of HIV-1 LTR by Amino Acid Analogs Requires NF-κB Binding Sites—We next investigated whether the two κB sites present in HIV-1 LTR were necessary for the activation of this promoter by amino acid analogs. To
Amino Acid Analogs Are Powerful Activators of NF-κB Transcription Factor—To investigate whether the activation of HIV-1 LTR or isolated κB promoter by amino acid analogs correlated with NF-κB activation, the nuclear redistribution of NF-κB subunits as well as the in vitro DNA binding of this factor were analyzed. It is seen in the immunoblot analysis presented in Fig. 4 that the p65/RelA subunit of NF-κB redistributed into the nucleus in HeLa cells treated with 15 mM azetidine. This phenomenon, which was already detectable after 1 h of treatment, gradually increased until 6 h of incubation with azetidine. Moreover, the nuclear redistribution of p65/RelA was reversible since it was no longer observed in cells allowed to recover for 4 h in the absence of azetidine. A similar observation was made when HeLa cells were treated with canavanine or when the other subunit of NF-κB, p50, was analyzed (not shown). In vitro DNA binding of NF-κB was investigated by electrophoretic mobility shift assays. To this end, nuclear extracts were prepared from HeLa cells either left untreated or exposed for 6 h to 5 or 15 mM of azetidine or canavanine. DNA binding assays were performed using a DNA probe encompassing the κB motif (see “Materials and Methods”). It is seen in Fig. 5 that azetidine or canavanine induced the formation of a specific protein/κB oligonucleotide complex that was not observed in untreated control cells. Competition experiments performed with an excess of nonradioactive κB oligonucleotide competed effectively for complex binding. Moreover, a supershifted band was observed when the reaction mixture was incubated with an antibody that recognizes the p65/RelA subunit of NF-κB to “κB” oligonucleotide.

Amino Acid Analogs Activate NF-κB by Photodynamic Inactivation of the Specific Binding of NF-κB to the κB Motif—It is seen in the immunofluorescence analysis presented in Fig. 6 that amino acid analogs did not modify the size of proteins that could result in their rapid degradation (17, 19). We then investigated whether treatments of HeLa cells by amino acid analogs altered the level of the p65/RelA, p50, and 1κBα subunits of NF-κB. It is seen in the immunoblot analysis presented in Fig. 6, that amino acid analogs did not modify the
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Fig. 6. Effects of amino acid analog treatments on the electrophoretic mobility and stability of the p65/RelA, p50, and IκB-α subunits of NF-κB. A and B, HeLa cells were treated with 15 mM azetidine or canavanine for 1, 2, 4, or 6 h, or were kept untreated (c). C and D, HeLa cells were treated with azetidine (C) or canavanine (D) during various time ranging from 5 min to 6 h. Control untreated cells (0) or control untreated cells kept in DMEM containing dialyzed fetal calf serum (dFCS/0) are presented. Equal amounts of total cellular extracts were separated by a 12.5% (p65 and p50) or a 15% (IκB-α) SDS-polyacrylamide gel electrophoresis. The cellular contents of NF-κB subunits p65/RelA (A), p50 (B), and IκB-α (C and D) were analyzed in immunoblots probed with antibodies that recognize specifically these proteins and revealed by ECL. Note that amino acid analogs did not alter the cellular content of p50 and p65/RelA, which contrasts with the induction of decreased levels of IκB-α by these treatments.

electrophoretic mobility and cellular content of p65/Rel A (Fig. 6A) or p50 (Fig. 6B). In contrast, treatments of HeLa cells by 15 mM of azetidine or canavanine altered IκB-α stability. Immunoblots probed with an antibody specific for IκB-α/MAD-3 (Fig. 6, C and D) show that azetidine (C) or canavanine (D) induced a drastic decrease of IκB-α level which began to be detectable after 60 min of treatment.

IκB-α is characterized by a short half-life (35, 67) which, in HeLa cells, was estimated by pulse-chase experiments followed by immunoprecipitation to be about 30–40 min (data not shown). Hence, experiments were performed to test whether the rapid disappearance of IκB-α observed above did not simply result from protein synthesis inhibition induced by amino acid analogs. To this end, HeLa cells were labeled with [35S]methionine/cysteine prior to and at different time periods during the treatment. It is seen in Fig. 7A, that, in our conditions of treatment with azetidine, the overall pattern of protein synthesis was not significantly altered, except for the enhanced synthesis of two high molecular weight proteins which are probably hsp70 and Grp78. These two proteins displayed different kinetics of activation; Grp78 expression was transient and peaked after 2 h of treatment while hsp70 was expressed later. A similar result was observed when cells were treated with canavanine (not shown). The level of IκB-α synthesis was next analyzed by immunoprecipitation of extracts of HeLa cells treated for 60 min with 15 mM of azetidine or canavanine and labeled with [35S]methionine/cysteine for the last 40 min of the treatment (see “Materials and Methods”). As seen in Fig. 7B, IκB-α was still synthesized in the presence of azetidine or canavanine, hence suggesting that these analogs were incorporated into this protein. However, in this case, the level of [35S]methionine/cysteine incorporation in IκB-α appeared less intense than in cells not treated with the analogs. This phenomenon probably reflects the stimulated degradation of IκB-α in amino acid analog-treated cells, since it is rather improbable that analogs will discriminate between the translation of specific mRNAs.

The possibility of an involvement of the proteasome pathway in the degradation of IκB-α, in amino acid analog-treated cells, was tested by studying the effect of a proteasome inhibitor on IκB-α stability, during azetidine treatments. It is seen in Fig. 8 that a 50-min preincubation of HeLa cells with 60 μM of the specific inhibitor of the proteasome chymotrypsin-like activity (PSI) (44, 68) completely abolished IκB-α degradation induced by azetidine. A similar effect was observed in cells treated with canavanine. This result therefore demonstrates that the chymotrypsin-like activity of the proteasome is probably responsible for the degradation of IκB-α in amino acid analog-treated HeLa cells.

Several reports have described the rapid appearance, in SDS-polyacrylamide gel containing 10% acrylamide, of a slow migrating phosphorylated form of IκB-α that precedes the degradation of this protein in response to TNF-α (44, 45, 47, 52, 69–71). This transient phosphorylation of IκB-α by amino acid analogs was therefore investigated. It is seen in Fig. 9 that azetidine treatments did not induce the appearance of a slow migrating phospho-isoform of IκB-α even if cells were incubated with 60 μM of the proteasome inhibitor PSI. By contrast, a 5-min treatment of HeLa cells with hydrogen peroxide did induce the appearance of a slow migrating IκB-α-phospho-isoform which was rapidly degraded except if cells were treated with PSI, a phenomenon which was already reported by others (44, 45, 47, 69–71).
Changes in IkB-α isoform composition in response to azetidine or canavanine treatments was further analyzed by two-dimensional immunoblot. This technique allows IkB-α to be resolved into two major isoforms; the acidic “b” phospho-isoform and the more basic “a” unphosphorylated isoform (52). Azetidine (Fig. 10A) or canavanine (not shown) treatments were not found to change the isoform pattern of IkB-α, hence, suggesting that this protein is not phosphorylated in response to treatment with these compounds. In contrast, a five minute treatment with 250 μM H₂O₂ drastically increased the level of the b isoform and concomitantly decreased the level of the a isoform (Fig. 10B) confirming that a large fraction of IkB-α is rapidly phosphorylated in response to hydrogen peroxide. These results, therefore strongly suggest that, in HeLa cells, amino acid analogs activate NF-κB, via IkB-α degradation by the proteasome, but apparently without the crucial step of IkB-α phosphorylation.

The Antioxidant PDTC Strongly Decreases the Transcriptional Activation of HIV-1 LTR by Amino Acid Analogs while the Reverse Is Observed in Case of Human hsp70 Promoter—Most NF-κB inducers have been reported to be inhibited by antioxidants, such as PDTC, implying that intracellular redox...
Fig. 9. One-dimensional immunoblot analysis of IκB-α isoforms in HeLa cells exposed to azetidine or hydrogen peroxide. HeLa cells were treated for various time periods with 250 μM hydrogen peroxide or 15 mM azetidine in the presence or not of 60 μM proteasome inhibitor PSI. Cells were harvested, and equal amounts of total cellular proteins were separated in SDS-PAGE containing 10% acrylamide as described by Traenckner et al. (44). The cellular contents of IκB-α isoforms was analyzed in immunoblot probed with IκB-α antibody and revealed by ECL as described under “Materials and Methods.” Note the absence of any slow migrating IκB-α phospho-isoform after amino acid analog treatments even in the presence of proteasome inhibitor, in contrast to the rapid appearance of IκB-α phospho-isoform (P-IκB-α) after H2O2 treatment.

Fig. 10. Two-dimensional immunoblot analysis of IκB-α isoforms in Hela cells exposed to azetidine or hydrogen peroxide. Total proteins of HeLa cells, treated or not with 15 mM azetidine (A) or 250 μM hydrogen peroxide (B) during various time periods were analyzed in two-dimensional immunoblot probed with anti-IκB-α antibody and revealed by ECL as described under “Materials and Methods.” As indicated, analysis was performed before (c) and after 15, 30, 60, 180, or 360 min of treatment with azetidine or before (c) and after 5, 15, 30, 45, or 60 min of treatment with hydrogen peroxide. The more acidic with apparent higher molecular weight IκB-α phospho-isoform is indicated as the b isoform. The less acidic and faster migrating IκB-α isoform is indicated as the a isoform. Note that azetidine treatments did not change the isoform pattern of IκB-α, in contrast to the rapid appearance of IκB-α b phospho-isoform induced by hydrogen peroxide.

or reactive oxygen species modulate the signal transduction that results in NF-κB activation (7, 72). We therefore investigated whether PDTC could modulate HIV-1 LTR transcriptional activation by amino acid analogs. To this end, HeLa cells, transiently transfected with pLTR-cat plasmid were first incubated for 1 h with 100 μM of PDTC before being exposed or not for 6 h to increasing concentrations of azetidine or canavanine. 24 h after these treatments, the amount of CAT produced was estimated by CAT-ELISA testing (see “Materials and Meth-
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Figure 11. Effect of the antioxidant PDTC on HIV-1 LTR activation by amino acid analogs. HeLa cells transiently transfected with an HIV-1 LTR (A and B)-dependent reporter cat construct were either left untreated or pretreated for 1 h with 100 μM PDTC. Subsequently, cells were treated for 6 h with the indicated concentrations of azetidine (A) or canavanine (B). Cells were then allowed to recover for 24 h before being harvested. C, control experiment performed with HeLa cells transiently transfected with hsp70 promoter-dependent reporter cat construct (p17-cat-neo). Azetidine or canavanine treatments in presence or not of PDTC were performed as in A and B. Determination of CAT concentration and presentation of the results are as in Fig. 1. The histograms shown are representative of three identical experiments; S.D. are presented (n = 3).

DISCUSSION

Amino Acid Analogs Activate HIV-1 LTR through a κB-dependent Mechanism—HIV-1 LTR is transcriptionally activated by several cellular stress including heat shock (6, 8–10). However, the molecular mechanisms underlying the thermal activation of HIV-1 LTR are still a matter of speculation. Indeed, heat stress, and to a larger scale, fever, are very complex stimuli that involve juxtaposition of multiple events and among them the induction of unfolded and denatured proteins (17). To test whether the accumulation of aberrantly conformed proteins could be a signal for HIV-1 LTR activation, we studied the effect of amino acid analog treatments on HIV-1 LTR. Amino acid analogs induce irreversible protein conformation and are potent activators of the heat shock promoter and of the unfolded protein response pathway (19, 41, 76, 77). We show here that azetidine and canavanine were about four times more powerful to activate HIV-1 LTR than the heat shock promoter. This difference in activation was reversed when cells were exposed to heat shock. Moreover, we have observed that the activation of HIV-1 LTR by amino acid analogs depended on the two κB sites located in the LTR and that a κB driven reporter gene was strongly activated by these analogs. These results therefore indicate that amino acid analogs are new members of the already impressive list of κB-mediated transcriptional activators. Besides, these findings suggest that accumulation of mal-folded proteins in the cell triggers κB-mediated transcription and that pathologies interfering with protein folding are probably inducers of κB-dependent gene expression.

Amino Acid Analogs Activate the Transcription Factor NF-κB—We have shown that treatments of HeLa cells with amino acid analogs induced the specific DNA binding and transcriptional activity of NF-κB. A similar activation was observed in an other human cell line (T47D cells). Since amino acid analogs can induce abnormal conformation of the proteins in which they are incorporated, it can be hypothesized that the accumulation of misfolded proteins represents a stimulus that triggers NF-κB activation. This hypothesis is strengthened by the fact that puromycin, an analog of aminoacyl tRNA which induces a premature release of polypeptide chains from the translation machinery, therefore creating abnormal polypeptides inside the cell, can induce κB-dependent transcriptional activity and NF-κB activation.2

Amino Acid Analogs Induce IκB-α Degradation without Any Prior Modification of the Distribution of Its Isoforms—We report here that amino acid analogs activate NF-κB through IκB-α degradation. This breakdown of IκB-α level is not induced by an inhibition of global protein synthesis nor of IκB-α synthesis, since we showed that, after 60 min of treatment with 15 mM azetidine, global protein synthesis was not altered and IκB-α was still synthesized. On the other hand, at that time of azetidine treatment, IκB-α level was drastically decreased, suggesting that the degradation of IκB-α was enhanced during the amino acid analog treatment. Stimulation-induced breakdown of IκB-α or basal turnover of this inhibitory subunit are both mediated by the proteasome (57). We show here that a specific proteasome inhibitor (PSI) completely abrogated IκB-α degradation. The Ca²⁺ chelator BAPTA-AM (75), we therefore incubated HeLa cells for 1 h with 20 μM of BAPTA-AM before amino acid analog treatment. This pretreatment was not found to prevent IκB-α-induced degradation (data not shown), hence suggesting that the oxidative stress generated by amino acid analogs was not due to an accumulation of mal-folded proteins, mediated by these compounds, in the ER.

2 C. Kretz-Remy and A.-P. Arrigo, unpublished results.
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Fig. 12. Inhibition of amino acid analog-induced IκB-α degradation by treatment with PDTC or glutathione peroxidase overexpression. A, HeLa cells were either left untreated or pretreated for 1 h with 100 μM PDTC. Subsequently, cells were treated during various time periods ranging from 15 min to 6 h with 15 μM azetidine. Equal amounts of total cellular proteins were analyzed by SDS-polyacrylamide gel electrophoresis and the cellular content of IκB-α was investigated by immunoblot analysis using an antibody specific to this protein. B, same as in A, but in this case, control (Hygro) or glutathione peroxidase overexpressing (GPx) human carcinoma T47D cells (see “Materials and Methods”) (52) were exposed to azetidine treatments. Note the inhibition of amino acid analog-mediated IκB-α degradation in HeLa cells treated with PDTC as well as in T47D cells overexpressing glutathione peroxidase.

The 60 min required to observe degradation of IκB-α by the proteasome probably reflects the time needed to incorporate amino acid analogs into proteins that have a relatively short half-life. Since amino acid analogs can induce abnormal protein degradation after azetidine treatment, suggesting an important role played by this protease. IκB-α level was even slightly enhanced in the presence of this inhibitor, confirming the role of the proteasome in IκB-α basal turnover (57). Moreover, this degradation of IκB-α is a slow phenomenon, which begins to be detectable after 1 h of treatment with 15 μM azetidine or canavanine, and is not preceded by a change in IκB-α isoform distribution, as shown by one- or two-dimensional immunoblot analysis. This suggests that amino acid analogs do not trigger IκB-α phosphorylation prior to its degradation. This phenomenon contrasts with the TNF-α- or H2O2-mediated rapid phosphorylation and subsequent degradation of IκB-α that has been reported by many authors (45, 47, 52, 69). Hence, one could hypothesize that IκB-α breakdown in the presence of amino acid analogs may result from an unbalanced basal turnover of this protein instead of an induced phosphorylation-ubiquitination-mediated breakdown. Indeed, IκB-α basal turnover does not appear to require any ubiquitine conjugation or phosphorylation event, but is still dependent on the proteasome (57).

The 60 min required to observe degradation of IκB-α by the proteasome probably reflects the time needed to incorporate amino acid analogs into proteins that have a relatively short half-life. Since amino acid analogs can induce abnormal protein degradation, the gradual accumulation of misfolded proteins may be a signal for IκB-α degradation. On the other hand, IκB-α is rich in proline and arginine (78) and has a short half-life of about 30–40 min (35, 67) (this study). Consequently, the azetidine or canavanine treatments used in this study probably poison IκB-α and generate aberrant conformation of this polypeptide. This phenomenon may directly stimulate the basal degradation of IκB-α by the proteasome. In comparison, the half-life of the two NF-κB subunits, p65 and p50, is very long (between 8 and 24 h) (35). Thus, although p65 and p50 are rich in arginine and proline (79, 80), their very long half-lives suggest that, in our experimental conditions, only a small fraction of these proteins is altered by amino acid analogs. These observations suggest that NF-κB activation by amino acid analogs is not a consequence of amino acid analog-poisoned p50 and p65 but rather results from the direct or indirect action of amino acid analogs on the short-lived protein IκB-α. Since NF-κB activation appears to be time-correlated with IκB-α degradation, it is difficult to decide whether or not amino acid analogs trigger the release of IκB-α from the NF-κB-IκB-α complex before IκB-α is degraded.

NF-κB Activation by Amino Acid Analogs Is Redox-dependent—Like most NF-κB inducers tested so far, the transcriptional activation of xB-driven genes by canavanine or azetidine was inhibited by the antioxidant PDTC. A similar observation was made when the experiment was carried out in T47D cells that overexpress glutathione peroxidase. Our experiments therefore suggest that amino acid analogs induce an intracellular oxidative stress that is essential for triggering NF-κB activation. In contrast, antioxidant conditions drastically enhanced the transcriptional activation of hsp70 promoter by amino acid analogs. Hence, while xB and hsp70 promoters are activated by several common inducers, the maximal inducibility of these two promoters appears inversely regulated by intracellular redox. It is not known how amino acid analogs generate oxidative stress. However, these compounds are known to trigger the synthesis of the ER protein chaperone Grp78/Bip and other glucose-related proteins (17, 23–25) through the unfolded protein response pathway (41, 76, 77). Indeed, in our conditions, the accumulation of Grp78, which reflects that of misfolded proteins in the ER, was transient and peaked after 2 h of treatment with the analogs, indicating that HeLa cells were under such an ER stress. Nevertheless, the unfolded protein response signal transduction pathway does not seem to involve the generation of reactive oxygen species (81–84). Hence, we tested whether an other type of ER stress caused by ER overload, which is known to generate oxidative stress, the so-called redox-dependent NF-κB ER to nucleus stress pathway (41), could be responsible for the pro-oxidative status induced by amino acid analogs. This ER overload results in a Ca2+ release from the ER that activates ROS producing...
enzymes such as cyclooxygenase and/or lipoxygenase and subsequently NF-κB (41, 74). Preincubation of HeLa cells with the intracellular Ca²⁺ chelator BAPTA-AM, which abolishes NF-κB induction by ER overload (75), was not found to prevent IκB-a induced degradation by amino acid analogs. Hence, the oxidative stress generated by amino acid analogs, which is necessary for IκB-a degradation by the proteasome, is probably not due to the effect mediated by amino acid analogs in the ER.

The Proteolytic Degradation of IκB-α Requires Pro-oxidant Conditions—In the case of NF-κB activation by hydrogen peroxide or TNF-α, IκB-α phosphorylation at serines 32 and 36 precedes (69–71) and is necessary for the rapid degradation of this protein by the proteasome (44, 45, 48–50). In this system of activation, IκB-α phosphorylation and degradation are abolished by antioxidants (42, 69) or the overexpression of the protease is regulated by redox. In this respect, it is interesting that, in unstressed T47D cells, glutathione peroxidase overexpression of this protein is also under the control of intracellular redox. It is, however, not yet known whether the activity of the IκB-α protease is regulated by redox. In this respect, it is interesting to note that the proteasome contains a phosphorylated subunit (85, 86) whose level of phosphorylation may represent a target that could be modulated by intracellular redox.

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