HIV-1 Accessory Protein Vpr Inhibits the Effect of Insulin on the Foxo Subfamily of Forkhead Transcription Factors by Interfering With Their Binding to 14-3-3 Proteins

Potential Clinical Implications Regarding the Insulin Resistance of HIV-1–Infected Patients

Tomoshige Kino,1 Massimo U. De Martino,1 Evangelia Charmandari,1 Takamasa Ichijo,1 Taoufik Outas,2 and George P. Chrousos1

HIV-1 accessory protein Vpr arrests host cells at the G2/M phase of the cell cycle by interacting with members of the protein family 14-3-3, which regulate the activities of “partner” molecules by binding to their phosphorylated serine or threonine residues and changing their intracellular localization and/or stability. Vpr does this by facilitating the association of 14-3-3 to its partner protein Cdc25C, independent of the latter’s phosphorylation status. Here we report that the same viral protein interfered with and altered the activity of another 14-3-3 partner molecule, Foxo3a, a subtype of the forkhead transcription factors, by inhibiting its association with 14-3-3. Foxo3a’s transcriptional activity is normally suppressed by insulin-induced translocalization of this protein from the nucleus into the cytoplasm. Vpr inhibited the ability of insulin or its downstream protein kinase Akt to change the intracellular localization of Foxo3a preferentially to the cytoplasm. This HIV-1 protein also interfered with insulin-induced co-precipitation of 14-3-3 and Foxo3a in vivo and antagonized the negative effect of insulin on Foxo3a-induced transactivation of a FOXO-responsive promoter. Moreover, Vpr antagonized insulin-induced suppression of the mRNA expression of the glucose 6-phosphatase, manganese superoxide dismutase, and sterol carrier protein 2 genes, which are known targets of insulin and FOXO, in HepG2 cells. These findings indicate that Vpr interferes with the suppressive effects of insulin on FOXO-mediated transcription of target genes via 14-3-3. Vpr thus may contribute to the tissue-selective insulin resistance often observed in HIV-1–infected individuals.

Diabetes 54:23–31, 2005

The HIV-1 protein Vpr, a 96–amino acid virion-associated accessory protein, is important for virus propagation in vivo and has multiple other functions (1–6). Vpr is packaged in significant quantities into viral particles (7,8) and is imported into the nucleus early after infection (9). Vpr participates in the nuclear translocation of the HIV-1 preintegration complex (10,11) and also functions as a coactivator of several steroid hormone receptors (12,13). Vpr is detected in sera of HIV-1–infected patients, and when it is administered extracellularly, it penetrates cell membranes, enters into the cytoplasm and nucleus of cells, and exerts its actions in both cytoplasmic and nuclear compartments. Thus, the biologic effects of Vpr may be exerted in neighboring or distant tissues that are not directly infected with the HIV-1 virus (14–16).

In addition to the above-indicated activities, Vpr efficiently arrests the host cell cell cycle at the G2/M boundary (17–19). To discover one or more molecules that support Vpr’s cell cycle–arresting activity, we recently performed yeast two-hybrid screening assays using wild-type and mutant Vpr molecules as baits (20). We found that Vpr physically interacted with 14-3-3 proteins (20), which regulate numerous cellular activities by changing the intracellular location and/or stability of “partner” molecules after binding to phosphorylated serine/threonine residues in special sequences of these molecules (21,22). Vpr bound to 14-3-3 at the COOH-terminal domain of the latter, outside the binding sites for the phosphorylated amino acids of partner proteins. In a recent study from our laboratory, Vpr supported the complex formation of 14-3-3 and one of its partner proteins, Cdc25C, independent of the phosphorylation state of the latter, and prevented
Cdc25C translocation into the nucleus (20). Thus, it seems that Vpr arrests the host cells at the G2/M phase of the cell cycle by retaining 14-3-3 and Cdc25C in the cytoplasm.

Infection with HIV-1 results in AIDS, which is characterized by profound defects in the immune system that lead to opportunistic infections and neoplastic processes (23). In addition, these patients frequently develop malnutrition/body weight loss and wasting, as well as growth retardation in children, states associated with paradoxically increased levels of serum glucose, triglycerides, cholesterol, and insulin (24–26). These pieces of evidence indicate that tissues of HIV-1–infected patients are characterized by reduced sensitivity to insulin, i.e., insulin resistance.

Insulin regulates diverse physiologic functions of cells and tissues, such as carbohydrate and lipid metabolism, protein synthesis, DNA replication, cell growth and differentiation, and inhibition of apoptosis (27). Binding of insulin to its receptor stimulates many signaling cascades via phosphorylation-mediated activations and activates several transcription factors that, finally, regulate expression of target molecules (28). Insulin-responsive genes have at least eight distinct consensus insulin-responsive sequences (IRSSs) in their promoter regions that positively or negatively respond to insulin stimuli (29). Consensus sequences, such as those of activator protein 1, Ets, E-box, and thyroid transcription factor 2, mediate positive transcriptional effects of insulin, whereas an element with the consensus sequence T(G/A)TTT(T/G)-(G/T), also referred to as the PEPCK-like motif, mediates the inhibitory effect of insulin on several insulin-responsive genes (29). The forkhead in human rhabdomyosarcoma (FKHR or Foxo1), one of the members of the FOXO subfamily of the forkhead proteins that share the forkhead DNA-binding domain and play diverse roles in developmental and metabolic functions, has recently been shown to bind this PEPCK-like motif (30).

Insulin regulates the activity of several FOXO proteins, such as Foxo3a (FKHR-L1) and Foxo4 (AFX), in addition to Foxo1 (FKHR) (31–33). In the absence of insulin, they are located in the nucleus, bind to their responsive promoters, and activate the transcription rate of their target genes, including the key gluconeogenesis enzyme PEPCK, the IGF-binding protein 1, and the glucose 6-phosphatase (G6Pase) enzyme, which dephosphorylates glucose to facilitate its excretion from the liver (29,31,34,35). FOXO proteins also regulate mRNA expression of the manganese superoxide dismutase and sterol carrier protein 2 genes, which are also responsive to insulin (36,37). The former plays an important role in the protection of tissues from oxidative stress (38), and the latter is involved in the intracellular transport of cholesterol and phospholipids and in the activation of enzymes involved in fatty acyl CoA transacylation (39).

Once insulin induces the phosphorylation of specific serine and threonine residues of these FOXO proteins via activation of Akt or protein kinase B, these phosphorylated amino acids create binding sites for 14-3-3; then, activation of Akt or protein kinase B, these phosphorylation-induced 14-3-3 dimers then mask the forkhead domain of the former, as well as its closely located nuclear localization signal; thus, binding of 14-3-3 inhibits access of FOXO to DNA and stimulates its translocation from the nucleus to the cytoplasm (42,43). Two nuclear export signals located in the NH2- and COOH-termini of Foxo1, respectively, influence the latter process (42). These pieces of evidence therefore indicate that FOXO proteins function as transcription factors suppressed by insulin, and their binding to 14-3-3 is a crucial step in insulin’s ability to exert its inhibitory actions. In parallel, insulin and/or Akt may also regulate FOXO-induced transcriptional activity independent of their effect through 14-3-3, for example, by directly affecting the nuclear export/import and DNA-binding of FOXOs or via other as yet unknown mechanisms (42,43).

Because Vpr binds 14-3-3 and changes its binding specificity to its partner protein Cdc25C, we hypothesized that Vpr might also modulate the binding activity of 14-3-3 to other partner molecules, the FOXO family proteins. We show that Vpr inhibits the association of 14-3-3 and Foxo3a (FKHR-L1) and antagonizes insulin’s effect on Foxo3a. Our results indicate that Vpr may be one of the viral factors that participate in the mechanism of insulin resistance seen in some HIV-1–infected patients.

**RESEARCH DESIGN AND METHODS**

**Plasmids.** pCDNAs3/Vpr and -VprR80A, which express wild-type and R80A mutant Vpr, respectively, were described previously (12). pCMV-FLAG-Vpr and -VprR80A, which respectively express FLAG epitope-tagged wild-type Vpr and R80A mutant Vpr, were also described previously (12). HA-FKHR-L1 (HA-Foxo3a), HA-FKHR-L1-T32, S253, S312A, and HA-AKT-ΔPH, which express the hemagglutinin epitope-tagged wild-type Foxo3a (FKHR-L1) and Foxo3a (FKHR-L1) mutant that harbors mutations replacing a threonine and serine located at amino acids 32, 253, and 312 to alanine, and constitutively active Akt were gifts from Dr. M. Greenberg (Harvard Medical School, Boston, MA). pCMV-FLAG-Cdc25C was a gift from Dr. J. Chou (University of Michigan Medical School, Ann Arbor, MI) (44). pGEX-4T3-Foxo3a and -Cdc25C were constructed by subcloning Foxo3a and Cdc25C cDNAs into pGEX-4T3 in an in-flame manner, respectively. pHook-1, pSV40-Bgal, and Bluescript SK (+) were purchased from Invitrogen (Carlsbad, CA), Promega (Madison, WI), and Stratagene (La Jolla, CA), respectively. A probe for the Northern blot analysis of the G6Pase gene was a gift from Dr. J. Guan (University of Michigan Medical School, Ann Arbor, MI) (44). pGEX-4T3–Foxo3a was constructed as described (40), fused to the hemagglutinin epitope (HA), and expressed together with GST-fused Foxo3a was detected under a Leica DM IRB inverted microscope (Leica, Palo Alto, CA). 3xIRS-Luc, which has the luciferase gene under the control of three FOXO-responsive elements, was a gift from Dr. K.L. Guan (National Institutes of Health, Bethesda, MD).

**Cell culture, transfection, reporter assays, and detection of EGFP fused Foxo3a.** Human cervical carcinoma HeLa cells and human hepatoma HepG2 cells were purchased from American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% of fetal bovine serum, 100 units/ml penicillin, 1 μg/ml streptomycin sulfate, and 25 mmol/l HEPES. Cells were transfected with either the Lipofectin or CaPO4 method, as described previously (12,13). For the experiments to detect EGFP, HeLa cells were seeded in 20-mm-diameter dishes 1 day before transfection and 0.3 μg per well of pEGFP-C1–Foxo3a and Vpr-expressing plasmids were used for the transfection. Cells were treated with 100 mmol/l insulin after 24 h of transfection or were transfected with 0.2 μg per well of HA-AKT-ΔPH to phosphorylate expressed Foxo3a. The EGFP fused Foxo3a was detected under a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany). Images were captured with a charge-coupled device camera (Shimazu, Kyoto, Japan) and analyzed with the Openlab package (Improvision, Coventry, U.K.). For reporter assays, HeLa cells were transfected with 1 μg per well of HA-FKHR-L1 (HA-Foxo3a) or HA-FKHR-L1-T32, S253, or S312A, and 1 μg per well of Vpr-expressing plasmids, together with 1.5 μg per well of 3xIRS-Luc and 0.5 μg per well of pSV40-Bgal. For keeping the same amounts of DNA, empty vectors or Bluescript SK (+) were co-transfected.
were used. Twenty-four hours after the transfection, cells were treated with 100 nmol/l insulin, and cell lysates were collected after an additional 24-h incubation. Luciferase and \(\beta\)-galactosidase activities were determined as described previously (12). Luciferase activity was divided by \(\beta\)-galactosidase activity to account for transfection efficiency.

**Coimmunoprecipitation assay.** HeLa cells were grown in 175-cm\(^2\) flasks and transfected with 15 \(\mu\)g of each indicated plasmid. Cells were treated with 100 nmol/l insulin 24 h after transfection. After an additional 24 h, cells were lysed with buffer that contained 50 mmol/l Tris-HCl (pH 8.0), 120 mmol/l NaCl, 0.5% NP-40, and 1 Tab/50 ml Complete tablets (Roche Molecular Biochemicals, Indianapolis, IN), and extracts were centrifuged at 400 \(g\) at 4°C for 10 min. Supernatants were collected to obtain the whole homogenates (20). They were incubated with anti–14-3-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which cross-reacts with multiple 14-3-3 proteins, at 4°C for 3 h. Protein-antibody complexes were subsequently harvested by adding protein A/G agarose PLUS (Santa Cruz Biotechnology) and were washed three times with the buffer that contained 20 mmol/l Tris-HCl (pH 8.0), 100 mmol/l NaCl, 0.5% NP-40, and 1 Tab/50 ml Complete tablets. Samples were separated on 12 or 16% SDS-PAGE gels and blotted to a nitrocellulose membrane, which was subsequently treated with anti-hemagglutinin antibody (Santa Cruz Biotechnology). Expressed Vpr was detected with anti-FLAG (M2) antibody (Sigma, St. Louis, MO) in whole homogenates. Inputs of hemagglutinin (HA)-Foxo3a and 14-3-3 were detected in 10% of whole homogenates. Foxo1 was detected in 10% whole homogenates by using anti-Foxo1 antibody (Santa Cruz Biotechnology) in Western blots. Phosphorylated Foxo1, Foxo3a, and Akt were also detected in Western blot by using anti-Foxo1, -Foxo3a, and -Akt, which specifically recognize these molecules that are phosphorylated at specific amino acids (Santa Cruz Biotechnology). The membranes were treated with secondary antibodies, and blotted proteins were visualized by the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to high-performance chemiluminescence films (Hyperfilm ECL; Amersham Pharmacia Biotech).

**In vitro binding assay.** \(^{35}\)S-labeled Vpr was generated by in vitro translation and tested for interaction with bacterially produced and purified GST-Foxo3a or -Cdc25C immobilized on glutathione-sepharose beads in buffer that contained 50 mmol/l Tris-HCl (pH 8.0), 50 mmol/l NaCl, 0.1% NP-40, 10% glycerol, and 0.1 mg/ml BSA at 4°C for 1.5 h. After vigorous washing with the buffer, proteins were eluted and separated on a 14% SDS-PAGE gel. Gels were fixed and exposed on film. Expression and purification of GST, and GST-Foxo3a and -Cdc25C were confirmed by running these proteins on a 4–20% SDS-PAGE gel and by visualizing with SimplyBlue SafeStain (Invitrogen).

**Northern blot analyses.** HepG2 cells were plated on 150-mm-diameter dishes 1 day before the experiments. They were transfected with pcDNA3-Vpr and pHook-1. Cells were subsequently treated with 100 nmol/l insulin or vehicle for 24 h. Transfection-positive cells were then collected through the epitope expressed from pHook-1 following the company’s instruction (13), and total RNA was isolated with TRIZOL (Invitrogen). Purified total RNA was
run on a formamide-denaturing gel and was blotted to a nylon membrane, which was subsequently treated with 32P-labeled probe directed to G6Pase or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The membrane was washed in SSPE buffers and was exposed to an X-ray film. Band density of G6Pase or GAPDH was measured by using the NIH Image software (National Institutes of Health, Bethesda, MD). The expression of G6Pase was corrected for the expression of GAPDH. Results represent the mean and SE of three independent experiments. Akt inhibitor 1L-6-hydroxymethyl-chio-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate was purchased from Calbiochem-Novabiochem (La Jolla, CA).

Quantitative real-time PCR for the evaluation of G6Pase, manganese superoxide dismutase, and sterol carrier protein 2 (SCP2) mRNA levels. HepG2 cells (1 × 10⁶) were cultured in serum-free medium for 48 h, plated on a 100-mm dish, and transfected with pCDNA3-Vpr wild-type or R80A, and pHook1. The cells were subsequently treated with 100 nmol/l insulin or vehicle for 24 h. Transfection-positive cells were then collected through the epitope expressed from pHook1 as per company instructions (13), and total RNA was isolated with TRIZOL (Invitrogen). The reverse transcription reaction was carried out using random hexamers with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA), as reported previously (45). For detecting mRNA levels of G6Pase, manganese superoxide dismutase (MnSOD) and SCP2 and control human acidic ribosomal phosphoprotein P0 (RPLP0), primer pairs (G6Pase: forward primer 5'-CTACTCTTTCCATCTTTCAG-3', reverse primer 5'-GAGTTAGGAAGCACTGATC-3'; MnSOD: forward primer 5'-CAGGCTGGCGCTTGAAGAAG-3', reverse primer 5'-GGTTCACCAGCAG-3'; SCP2: forward primer 5'-GAAGGACGGTTCCAACCAG-3', reverse primer 5'-GGTTCACCAGCAG-3'; RPLP0: forward primer 5'-CGCGACCTGGAAGTCCAACT-3', reverse primer 5'-CCATCACGACCACAGCCTTC-3') were used. The real-time PCR, consisting of the heat activation of the Taq polymerase (at 95°C for 10 min) and the subsequent 62 PCR cycles (denaturing at 95°C for 15 s, annealing/extension at 62°C for 1 min) was performed in triplicate using the SYBR Green PCR Master Mix (Applied Biosystems) in an ABI PRIZM 7700 SDS lightcycler (Applied Biosystems). Obtained threshold cycle values of G6Pase, MnSOD, and SCP2 were normalized for those of RPLP0, and their relative mRNA expressions were shown as fold induction over the baseline. The dissociation curves of obtained threshold cycle values of G6Pase, MnSOD, and SCP2 were normalized for those of RPLP0, and their relative mRNA expressions were shown as fold induction over the baseline. The dissociation curves of used primer pairs showed a single peak, and samples after PCRs had a single expected DNA band in an agarose gel analysis (data not shown).

RESULTS

Vpr inhibits insulin- and Akt-induced cytoplasmic translocation of EGFP-Foxo3a. To test our hypothesis, we first examined the effects of Vpr on the nucleus to cytoplasm translocation of EGFP-Foxo3a induced by insulin in HeLa cells (Fig. 1A). In the absence of insulin, the majority of cells had EGFP-Foxo3a in the nucleus with minimal distribution in the cytoplasm (Fig. 1A, top left). Coexpression of Vpr did not affect the subcellular localization of EGFP-Foxo3a (Fig. 1A, bottom left). Addition of 100 nmol/l insulin, however, strongly shifted the intracellular localization of EGFP-Foxo3a from the nucleus to the cytoplasm (Fig. 1A, top right), whereas addition of Vpr moderately retained EGFP-Foxo3a in the nucleus (Fig. 1A, bottom right). These results indicate that Vpr antagonized insulin’s effect on the nucleus to cytoplasm translocation of Foxo3a.

We next used a constitutively active form of an Akt mutant instead of insulin, which directly phosphorylates Foxo3a, bypassing several intermediate phosphorylation-dependent reactions induced by insulin (Fig. 1B). Expression of this mutant Akt induced cytoplasmic redistribution of EGFP-Foxo3a in a manner similar to insulin (Fig. 1B, top right). Vpr also antagonized this effect of Akt on EGFP-Foxo3a localization, partially retaining it in the nucleus (Fig. 1B, bottom right).

We examined >100 cells that were cotransfected with EGFP-Foxo3a and Vpr-expressing plasmids and treated with 100 nmol/l insulin and categorized them into five groups that have different distribution patterns from com-

FIG. 2. A: Vpr inhibits the ability of insulin to induce 14-3-3 and Foxo3a association in HeLa cells. HeLa cells were transfected with HA-FKHR-L1 (HA-Foxo3a) and/or pCMV-FLAG-Vpr and pHook1. HA-Foxo3a/14-3-3 complexes were coprecipitated with anti-hemagglutinin antibody and blotted with anti-14-3-3 antibody. Expression of FLAG-Vpr, HA-Foxo3a, and 14-3-3 was tested in 10% of whole homogenates, which were used for the coprecipitation reactions. Phosphorylated forms of Foxo3a (Thr 32), Akt (Thr 308), and Akt (Ser 473) were also detected in 10% whole homogenates in Western blots. B: Vpr WT did not interfere with association of 14-3-3 and Foxo3a in HeLa cells. HeLa cells were transfected with HA-FKHR-L1 (HA-Foxo3a) and/or pCMV-FLAG-Vpr WT or -VprR80A and pHook1. HA-Foxo3a/14-3-3 or Vpr/14-3-3 complexes were coprecipitated with anti-hemagglutinin antibody or anti-FLAG antibody, respectively, and blotted with anti-14-3-3 antibody. Expression of FLAG-Vpr, HA-Foxo3a, and 14-3-3 was tested in 10% of whole homogenates, which were used for the coprecipitation reactions. C and D: Vpr does not bind to Foxo3a in vitro. In vitro-translated and 35S-labeled Vpr was mixed with bacterially produced and purified GST-Foxo3a or -Cdc25C immobilized on glutathione-sepharose beads, and their interaction was tested (C). Purified GST, GST-Cdc25C, and -Foxo3a were also run on a 4–20% SDS-PAGE and visualized with SimplyBlue SafeStain (D).
complete nuclear localization to complete cytoplasmic localization. We demonstrated cell numbers in each group as percentage of the total numbers of examined cells (Fig. 1C). In this analysis, the spectrum of cells that demonstrated different EGFP-Foxo3a distribution was from the cells that showed complete nucleus versus cytoplasm localization in the absence of insulin, consistent with the previous experiment. Addition of 100 nmol/l insulin strongly shifted EGFP-Foxo3a from the nucleus into the cytoplasm. Expression of wild-type Vpr weakly shifted localization of EGFP-Foxo3a in the nucleus in the absence of insulin. In the presence of insulin, however, Vpr expression partially retained EGFP-Foxo3a in the nucleus, suggesting that Vpr moderately antagonized insulin's effect on Foxo3a subcellular distribution. In contrast to wild-type Vpr, VprR80A mutant, which is defective in binding to 14-3-3 (20), did not affect the nucleus-to-cytoplasm translocation of EGFP-Foxo3a induced by insulin.

Vpr inhibits insulin-induced association of 14-3-3 and Foxo3a in vivo. We previously demonstrated that Vpr through its COOH-terminal portion binds 14-3-3 outside the phosphopeptide-binding pocket of the latter located COOH-terminal (20). Because Vpr inhibited insulin-induced nucleus-to-cytoplasm translocation of EGFP-Foxo3a, we hypothesized that binding of Vpr to 14-3-3 might interfere with association of 14-3-3 and FOXO proteins induced by insulin. We thus examined this possibility in a coimmunoprecipitation assay in HeLa cells (Fig. 2A). In the absence of insulin, Foxo3a was coprecipitated with 14-3-3 (Fig. 2A, top gel, lane 1). Addition of 100 nmol/l insulin increased the association of 14-3-3 and Foxo3a (Fig. 2A, top gel, lane 2). However, once Vpr was coexpressed, this viral protein inhibited the coprecipitation of 14-3-3 and Foxo3a that was induced by insulin (Fig. 2A, top gel, lane 4). In control studies, insulin stimulated phosphorylated Foxo3a (Fig. 2A, third top gel) and upstream Akt (Fig. 2A, bottom three gels), indicating that Vpr did not inhibit the Foxo3a/14-3-3 association by suppressing phosphorylation reactions on these molecules induced by insulin. In addition, VprR80A, which cannot bind 14-3-3, did not affect the association of these two molecules in contrast to the wild-type Vpr (Fig. 2B). Finally, Vpr did not bind Foxo3a in vitro (Fig. 2C). These results further strengthen the possibility that Vpr interfered with the association of 14-3-3 and Foxo3a via direct binding to 14-3-3.

Vpr antagonizes the negative effect of insulin on the transcriptional activity of Foxo3a. We next examined the effects of Vpr on Foxo3a-induced transactivation of a responsive promoter to address the functional significance of the observed inhibitory effect of Vpr on the insulin-induced association of Foxo3a and 14-3-3 (Fig. 3). We used 3xIRS-Luc, which contains three FOXO-responsive elements that drive the luciferase reporter gene, as a reporter plasmid. Overexpression of Foxo3a stimulated this promoter activity by fourfold, and addition of 100 nmol/l insulin suppressed Foxo3a-stimulated transactivation by 60%. Wild-type Vpr reduced insulin-induced inhibition of the Foxo3a-stimulated transcription, whereas it did not have a discernible effect on the basal activity of the promoter. VprR80A, however, failed to antagonize insulin-induced suppression of the Foxo3a activity. A Foxo3a mutant (Mut), which has amino acid substitutions at threonine 32 and serines 253 and 312 to alanines and, thus, is unable to bind 14-3-3, stimulated the promoter activity in an insulin-independent manner. Vpr did not affect the transcriptional activity of this mutant Foxo3a. These results are consistent with the observation obtained in the subcellular localization study and the coimmunoprecipitation experiments, in which Vpr respectively suppressed cytoplasmic redistribution of EGFP-Foxo3a and inhibited the association of Foxo3a and 14-3-3 induced by insulin. However, Vpr might also affect FOXO-induced transcriptional activity by a mechanism(s) independent of 14-3-3. Indeed, insulin and Akt may regulate FOXOs in multifaceted ways not all fully understood (42,43).

Vpr antagonizes insulin’s effect on the expression of the G6Pase mRNA and other two insulin/FOXO-responsive genes in HepG2 cells. We examined the effect of Vpr on the expression of endogenous G6Pase mRNA, a gene whose transcription is stimulated by FOXO proteins and, thus, is negatively regulated by insulin (29). We transfected HepG2 cells with the Vpr-expressing plasmids, harvested transfection-positive cells, and examined G6Pase mRNA expression in Northern blots. Expression of Vpr did not have an obvious effect on the G6Pase mRNA abundance, whereas 100 nmol/l insulin suppressed it by 65%. Addition of Vpr expression with the same concentration of insulin almost reversed the suppressive effect of insulin on the G6Pase mRNA expression (Fig. 4A, top two gels).
the same experiment, Foxo1, which is a major FOXO protein in HepG2 cells, was phosphorylated at threonine 24 in Western blots (Fig. 4A, bottom two gels). Mean ± SE values of the G6Pase expression corrected for the expression of GAPDH from three independent experiments are shown in Fig. 4B. In this cell line, an Akt kinase inhibitor 1L-6-hydroxymethyl-chio-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate reversed insulin-induced suppression of G6Pase mRNA expression in Northern blots and suppressed phosphorylation of Foxo1 in Western blots (Fig. 4C), indicating that insulin, at least in part, impaired G6Pase mRNA expression by inhibiting Foxo activity in this cell line. Taken together, these findings indicate that Vpr disrupts insulin-induced regulation of G6Pase gene expression by altering the interaction of Foxo with 14-3-3. However, it is possible that Vpr inhibits some of insulin’s effect on G6P gene expression via non–14-3-3–mediated mechanisms.

Because there are several conflicting reports indicating limited effect of FOXO proteins on G6Pase expression (31,46), we also tested Vpr on two other Foxo-responsive genes, MnSOD and SCP2, using real-time PCR in the same HepG2 cells (Fig. 4D). Expression of wild-type Vpr or the R80A mutant did not affect mRNA expression of G6Pase, MnSOD, or SCP2, whereas addition of insulin significantly suppressed it. Wild-type Vpr strongly attenuated the negative effect of insulin on their mRNA expression of these genes, whereas the 14-3-3 binding–defective Vpr R80A mutant exerted no such effects. Taken together, our results suggest that Vpr suppresses insulin’s effect on FOXO-induced expression of endogenous genes, such as those of G6Pase, MnSOD, and SCP2, possibly by inhibiting insulin-induced association of 14-3-3 and FOXO proteins in HepG2 cells.

**DISCUSSION**

In the present study, we investigated the effect of HIV-1 Vpr on the insulin-induced modulation of Foxo3a activity. We demonstrated that Vpr antagonized both the insulin- and Akt-induced translocation of Foxo3a from the nucleus to the cytoplasm. It also inhibited the association of Foxo3a with 14-3-3 induced by insulin in a coimmunoprecipitation assay. Furthermore, Vpr antagonized the negative effect of insulin on Foxo3a-induced transactivation of a responsive promoter and also inhibited the suppressive effect of insulin on the mRNA expression of the endogenous G6Pase, MnSOD, and SCP2 genes in HepG2 cells.
Because the VprR80A mutant, which is defective in binding to 14-3-3, lost all of these activities and because wild-type Vpr inhibited insulin-induced coprecipitation of 14-3-3 and Foxo3a, our results strongly suggest that Vpr inhibits binding of 14-3-3 to phosphorylated FOXO proteins and, thus, antagonizes insulin’s negative regulation of their activity. It is still possible, however, that Vpr regulates insulin/FOXO-responsive genes by 14-3-3–bypassing mechanisms (42,43,46).

The above effects of Vpr may be relevant to several biologic changes observed in HIV-1–infected lymphocytes and monocytes or nonimmune tissues, such as adipose tissue, that are primary targets of HIV-1 (47). However, the majority of insulin-responsive tissues, such as muscle and liver, have not been reported to be infected directly with HIV-1. The latter may not be necessary for the metabolic actions of Vpr, which is expressed and secreted by host cells infected by HIV-1 and is detected in extracellular fluids, such as plasma and cerebrospinal fluid, of HIV-1–infected patients (14). This is corroborated by extracellular administration of synthetic Vpr, which readily penetrates the cell membrane and causes cell cycle arrest or exerts other effects in HIV–uninfected cells (15,16), indicating that Vpr may also act as a paracrine or endocrine “hormone,” acting on neighboring or distant cells that are not infected with HIV-1.

AIDS patients frequently develop a pathologic state known as AIDS-associated insulin resistance and lipodystrophy syndrome, especially when given long-term therapy with anti-HIV drugs, such as protease inhibitors and nucleotide/nonnucleotide reverse transcriptase inhibitors (26). AIDS patients who have this syndrome have carbohydrate intolerance and dyslipidemia and may also develop overt diabetes, along with their cardiovascular sequelae, particularly atherosclerotic coronary artery disease (24,25,48–50). The cause of this syndrome is not clear but seems to be multifactorial. The HIV-1 infection itself seems to contribute to the development of these pathologic changes or increases the vulnerability of patients to develop the syndrome upon uptake of anti–HIV-1 drugs; some AIDS patients develop the characteristic features and metabolic disturbances of the syndrome before treatment (26,51,52). Our results suggest that Vpr might contribute to the insulin resistance of AIDS patients through both its FOXO-related insulin action blockade and its previously described glucocorticoid receptor coactivator activity (12,13). A recent report indicated that FOXO plays an important role in the adipocyte differentiation (53); thus, it is also possible that Vpr influences development of lipodystrophic phenotype seen in this syndrome by modulating FOXO activity.

Vpr inhibited insulin-induced association of 14-3-3 and Foxo3a in a coimmunoprecipitation assay. This indicates that binding of Vpr to 14-3-3 interferes with the interac-

---

**Fig. 4. Continued.**

![Diagram](image-url)
tion of phosphorylated serine and threonine residues of Foxo3α to a phosphopeptide-binding pocket located in 14-3-3. Vpr binds 14-3-3 at the COOH-terminal portion of the latter, outside the phosphopeptide-binding site, including the eighth α-helix of 14-3-3 (20). Because this portion of 14-3-3 also plays a role in the creation of the phosphopeptide-binding cleft, possibly by stabilizing the structure of 14-3-3 (54,55), it seems that Vpr reduces the binding activity of 14-3-3 to the phosphorylated serine and threonine residues of FOXO proteins. In contrast, we previously reported that Vpr facilitated binding of 14-3-3 and Cdc25C independent of the phosphorylation state of Cdc25C (20). These results indicate that Vpr influences the binding activity of 14-3-3 to its partners in different directions, depending on the partner molecule with which it associates.

In addition to the forkhead transcription factors, 14-3-3 proteins influence the function of numerous proteins involved in growth factor, hormone, and cytokine signaling, as well as apoptosis, and cell cycle regulation, such as IRS-1, Raf-1, MEKK1, Bcr, calmodulin/calmodulin kinase, protein kinase C, c-Cbl, BAD, and NFAT, through direct binding to their phosphorylated serine/threonine residues (21,56). Modulation of the activities of these proteins by Vpr might contribute to the development of other pathologic manifestations observed in HIV-1-infected patients.

We conclude that the HIV-1 accessory protein Vpr counteracts the negative regulation of insulin on the FOXO subfamily of the forkhead proteins possibly by inhibiting association between these proteins and 14-3-3. Our results may be relevant to pathogenetic mechanisms that account for the development of insulin resistance and related metabolic disturbances observed in HIV-1–infected individuals.

ACKNOWLEDGMENTS

We thank Drs. G.N. Pavlakis, A. Gragerov, J. Nakae, and D. Accili for insightful comments; Drs. M. Greenberg, K.L. Guan, and J. Chou for kindly providing plasmids; and K. Zachman and L. Chheng for excellent technical assistance.

REFERENCES


45. De Martino MU, Bhattacharyya N, Alesci S, Ichijo H, Chrousos GP, Kino T: The glucocorticoid receptor (GR) and the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) interact with and mutually affect each other’s transcriptional activities: implications for intermediary metabolism. Mol Endocrinol 18:820–833, 2004


