HHR23A, the Human Homologue of the Yeast Repair Protein RAD23, Interacts Specifically with Vpr Protein and Prevents Cell Cycle Arrest but Not the Transcriptional Effects of Vpr

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Yeast two-hybrid selection of proteins interacting with human immunodeficiency virus type 1 Vpr identified HHR23A, a human homologue of the yeast DNA repair protein RAD23, as a specific interactor. A small 57-amino-acid C-terminal portion of HHR23A was sufficient for Vpr interaction. When introduced into human cells by transfection, full-length HHR23A or its C-terminal fragments were able to alleviate Vpr-induced cell cycle arrest, suggesting that HHR23A may participate in the pathway leading to G2 arrest by Vpr. We have also examined the effects of HHR23A on the recently identified transcription coactivator function of Vpr. The two Vpr functions are independent, since we have identified mutants lacking either the cell cycle arrest or the coactivator function. Our analysis showed that excess of HHR23A does not affect the coactivator function of Vpr, while it affects the cell cycle arresting function. Therefore, a simple sequestering model for Vpr in the presence of excess HHR23A is not supported. We propose that the interaction of HHR23A with Vpr may affect specifically pathways leading to cell cycle regulation.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) encodes several accessory proteins in addition to the usual complement of retroviral structural proteins (for reviews, see Miller and Sarver, 1997; Pavlakis, 1996; Trono, 1995). One accessory protein with a role not well understood is Vpr, a 96-amino-acid protein found both in association with virions through its interaction with Gag p6 (Lavallee et al., 1994; Paxton et al., 1993) and in the nucleus of infected cells (Heinzinger et al., 1994; Wang et al., 1994). Several functions have been proposed for Vpr, including nuclear import of the preintegration complex (Heinzinger et al., 1994), induction of cell growth arrest and differentiation (He et al., 1995; Dowett et al., 1995; Levy et al., 1993; Re et al., 1995; Rogel et al., 1995), and transactivation of cellular genes (Cohen et al., 1990). Vpr is clearly dispensable for viral replication in T cell lines, but growth of vpr− molecular clones of HIV-1 in macrophages is severely delayed (Balliet et al., 1994; Hattori et al., 1990).

Although it is not clear which of the above-mentioned effects makes Vpr important for virus propagation, some aspects of HIV-1 pathogenesis may be caused by a disturbance of cells by Vpr. Expression of Vpr alone was shown to stop progression of the cell cycle (Dowett et al., 1995; Re et al., 1995), arresting cells in G2/M phase, and to cause differentiation of a rhabdomyosarcoma cell line (Levy et al., 1993). Substantial amounts of Vpr were found in the medium of infected cultures and in the serum of infected patients. This extracellular Vpr may exert at least some of its effects on uninfected cells, which may alter their ability to be infected and/or may contribute to HIV-1 pathogenesis. Extracellular Vpr was implicated in the activation of HIV-1 replication in vivo and in the control of latency (Levy et al., 1994). HIV-2 and simian immunodeficiency virus (SIV) express a similar protein, Vpx, in addition to Vpr (Tristem et al., 1992). Neither protein was essential for AIDS progression in infected macaques, but a double mutant lacking both Vpr and Vpx was severely attenuated (Gibbs et al., 1995). Because Vpr exerts many of its effects alone rather than in the context of the viral infection, it acts through certain cellular targets. This communication describes the identification of one such target, HHR23A, the human homologue of the yeast repair protein RAD23.

RESULTS AND DISCUSSION

To reveal intracellular interactions responsible for some of the reported Vpr effects, we used a version of the yeast two-hybrid system (Finley and Brent, 1995) with LexA-Vpr fusion as a bait. Numerous clones able to activate both reporter genes in the galactose-dependent manner (see Materials and Methods) were picked up in
a primary screen. To find candidate proteins interacting with Vpr, but not with other LexA fusions, we used the interaction mating test (Finley and Brent, 1994), in which yeast strains containing different library plasmids were mated with strains of the opposite mating type containing either Vpr or other HIV-1 protein fusion baits (Fig. 1). We were interested in proteins interacting specifically with Vpr. Only about 10% of the clones that were picked up in the primary screen passed this last test. Sequencing followed by the BLAST search of the available sequence databases showed that all the inserts coding for proteins demonstrating specific interaction with Vpr came from only two genes. Here we present our analysis of the interaction with the product of the one gene, HHR23A. The significance (if any) of Vpr's interaction with the second gene product is under investigation and will be described elsewhere. HHR23A (Masutani et al., 1994; van der Spek et al., 1996a) is the human homologue of the yeast excision-repair gene RAD23 (Mueller and Smerdon, 1996; Watkins et al., 1993). We identified four independent Vpr-interacting fusions of various sizes containing the C-terminus of the protein (Fig. 2). The shortest fusion shows that the Vpr-interacting part of the protein is carried within 57 C-terminal amino acids, roughly corresponding to the conserved internal repeat of RAD23 (van der Spek et al., 1996b). Both Vpr and HHR23A are localized to the cell nucleus (van der Spek et al., 1996a), making their interaction potentially relevant.

To verify that the identified interaction takes place in mammalian cells and has biological significance, we expressed HHR23A or its 57-amino-acid C terminal fragment in human cells together with Vpr (Fig. 3). Vpr expression under the control of CMV promoter, or in the context of the NL4-3 HIV-1 molecular clone, efficiently increased the fraction of cells in the G2/M phase of the cell cycle. Cotransfection of plasmids coding for either full-length HHR23A or a 57-amino-acid C-terminal fragment of the protein significantly reduced this Vpr effect. In addition, the 168-amino-acid fragment of HHR23A (corresponding to clone HP15) was tested with similar results (data not shown). Expression of GFP in the Vpr-

FIG. 1. Mating test for the Vpr-interaction specificity. Haploid bait strains are derivatives of RFY206 (Mata his3 leu2 lys2 ura3 trp1) transformed with the lacZ reporter plasmid pSH18-34 (URA3') and the indicated bait-expressing plasmids (HIS3', based on vector pEG202), and are streaked in horizontal lines. "Control" represents a preexaminied LexA fusion which activates transcription. Haploid prey strains derived from EGY48 (Mata his3 leu2::3Lexop-LEU2 ura3- trp1 LYS2) contain individual plasmids from the cDNA acid-fusion library in vector pJG4-5 and are streaked in vertical lines. Diploids forming in the intersections were tested for lacZ activity (top panel) and ability to grow without leucine (bottom panel). Arrows indicate clones that we considered specific for the interaction with Vpr.
expressing cells did not result in changes in the cell cycle profile, indicating that the effects of HHR23A on alleviation of the G2 cell cycle arrest caused by Vpr were specific. We can draw the following conclusions from these experiments. First, the interaction between Vpr and HHR23A detected in the yeast two-hybrid screen also takes place in human cells and is independent of LexA and acidic activator parts of the yeast constructs. Second, the interaction of the overproduced HHR23A with Vpr prevents the latter from blocking the cell cycle. Third, overproduction of HHR23A (or its fragments) by itself does not affect the cell cycle.

There are two possible explanations for the observed reduction of the Vpr-induced cell cycle effect by HHR23A. The simplest explanation is that the interaction of Vpr with endogenous HHR23A mediates cell cycle arrest and that HHR23A overproduction in excess of Vpr overcomes this effect. The other possibility is that HHR23A-Vrp binding prevents Vpr from interacting with another cellular target responsible for the cell cycle block. We favor the first explanation, however, because the 57-amino-acid C-terminal fragment of HHR23A, being considerably less bulky, is still effective in reversing Vpr-induced cell cycle arrest. Assuming that this fragment occupies the site that Vpr uses for binding to its cellular target responsible for cell cycle arrest, the simplest explanation is that this target is HHR23A itself. We hope to distinguish between these two possibilities by analyzing the cell cycle effect of Vpr mutants unable to bind HHR23A. We are currently selecting such mutants.

The possibility that HHR23A prevents Vpr action by titrating it out, or sequestering it somewhere in the cell, or causing Vpr degradation could be addressed by following HHR23A action on any effect of Vpr distinct from the cell cycle arrest. Recently Vpr has been proposed to act as a coactivator of the glucocorticoid receptor (Kino et al., 1998). Vpr interacts directly with the glucocorticoid receptor in vitro and is coprecipitated by anti-glucocorticoid receptor antibodies in extracts of glucocorticoid-treated cells, suggesting that it exerts its effects within the glucocorticoid-induced transcription initiation complex. Vpr contains the signature motif LXXLL that is also present in nuclear receptor coregulators, such as SRC-1 and CBP/p300, and is responsible for their interaction with the glucocorticoid and other nuclear receptors (Heery et al., 1997; Torchia et al., 1997). We used Vpr mutants to show that glucocorticoid receptor coactivation and cell cycle arrest are, indeed, two separate functions. Mutation L64A in the coactivator signature motif disrupts the ability of Vpr to influence transcription of glucocorticoid-responsive genes and makes this mutant a dominant negative inhibitor of the native protein (Kino et al., 1998). The same mutation does not influence the ability of Vpr to block the cell cycle (Fig. 4). Mutation R80A has a reciprocal effect: it prevents the cell cycle block (as also shown previously by others (Di Marzio et

FIG. 2. Independent clones containing fragments of HHR23A isolated by their ability to interact with Vpr. Numbers correspond to the full-length HHR23A mRNA (marked HHR23A). Boxes correspond to open reading frames. Gray boxes at the N-termini of clones isolated from the cDNA library represent the vector part of a protein fusion, including the nuclear localization signal, the B42 activation region, and the influenza hemagglutinin epitope tag. The upper lane represents (approximately in scale) the domain organization of the protein, showing the positions of the ubiquitin-like N-terminal part, internally repeated sequences, and proline-, alanine-, serine-, and threonine-rich (PAST) regions (van der Spek et al., 1996b).
al., 1995)) but does not affect GR coactivator activity (Fig. 4). Remarkably, HHR23A weakly increased the ability of Vpr to stimulate glucocorticoid receptor (Fig. 5), while it dramatically suppressed the Vpr-induced cell cycle arrest. Therefore, HHR23A does not block Vpr activity in general, but specifically interferes with its cell cycle action (compare Figs. 3 and 5). Western blot analysis also showed that HHR23A does not cause Vpr degradation (Fig. 3, insert).

The role of cell cycle arrest in HIV-1 expression is not clear. The following facts, however, argue in favor of some important function for the arrest. Vpr is conserved among all primate lentiviruses, and its ability to cause cell cycle arrest is also conserved. This property, however, is species specific, with Vpr of a particular virus being more effective in blocking cells of its natural host (Stvahil et al., 1997). HIV or SIV isolated from chronically infected cell lines is vpr−; otherwise, infected cells would be unable to divide (Rogel et al., 1995). Introduction of such a virus in animals results in selection of vpr+ virus (Lang et al., 1993), indicating that the Vpr function is of clear advantage in vivo.

Several approaches have been used to identify the cellular targets of Vpr. Protein bands were identified by coimmunoprecipitation with Vpr or by binding to immobilized Vpr (Rafaelli et al., 1995; Zhao et al., 1994). Neither of these putative targets was functionally characterized, but one of them was coimmunoprecipitated with Vpr and the activated glucocorticoid receptor complex (Rafaeli et al., 1995). As mentioned above, glucocorticoid receptor itself is a Vpr-interacting protein (Kino et al., 1998). Use of the yeast two-hybrid system by others led to the identification of a repair enzyme uracil-DNA-glycosylase (UNG), as a Vpr-interacting protein (Bouhamdan et al., 1996). Although this binding was confirmed in vitro, it did not inhibit UNG activity and its functional importance for Vpr action was not demonstrated. Vpr-induced cell cycle arrest is independent of this interaction (Selig et al., 1997).

The data reported in this paper implicate HHR23A as
a potential mediator of Vpr-induced cell cycle arrest. The yeast homologue of HHR23A, Rad23, is a UV-inducible protein (Madura and Prakash, 1990) required for efficient nucleotide-excision repair, both general and transcription-coupled (Guzder et al., 1995; Mueller and Smerdon, 1996; Wang et al., 1997). It has been shown to promote complex formation between transcription factor TFIIH and the DNA damage recognition protein Rad14 (Guzder et al., 1995). Both human and mouse homologues have been cloned, and each species has two homologous genes, named HHR23A and HHR23B. It is not clear whether they have distinct cellular functions (Masutani et al., 1994; van der Spek et al., 1996b). Part of the mammalian Rad23B is found in a complex with xeroderma pigmentosum group C protein (XP-C) (van der Spek et al., 1996a) and is stimulatory for the nucleotide excision repair activity of XP-C in vitro (Sugasawa et al., 1996). Yeast, mouse, and human Rad23 proteins share the following features (see Fig. 2): An 80-amino-acid ubiquitin-like domain present at the N-terminus, which is essential for biological function of yeast Rad23 (Watkins et al., 1993). It is followed by a nonconserved region composed mainly (86%) by proline, alanine, serine, and threonine (PAST domain). The rest of the protein contains two copies (one at the C-terminus) of an internally repeated 50-amino-acid element. This internally repeated sequence is fully conserved between human and mouse and is similar to a C-terminal extension of the bovine ubiquitin-conjugating enzyme E2(25K) (van der Spek et al., 1996b). It is the C-terminal copy of this repeated sequence that overlaps with the Vpr-interacting region of the protein. Provided there is a high similarity of the C-terminal copy of the internal repeat between HHR23A and B, it is not surprising that Vpr also interacts with HHR23B (Withers-Ward et al., 1997), although only HHR23A clones were picked up by the two hybrid screen.

If HHR23A is indeed the mediator of Vpr-induced cell cycle arrest, what may be the possible mechanism? One option is that by binding the repair protein, Vpr mimics DNA damage and triggers a checkpoint mechanism (for a review, see Elledge, 1996) blocking cells in G2. There is circumstantial evidence supporting such a view (Poon et al., 1997). Other workers argue, however, that the mechanism of Vpr-induced arrest is different from the DNA damage checkpoint (Bartz et al., 1996) with some cells accumulating DNA to polyploid levels, although the data are also indirect. Another possibility is indicated by studies of yeast RAD23. Inactivation of this protein together with DSK2, another protein that also has an ubiquitin-like N-terminus, prevents proper duplication of spindle pole bodies (Biggins et al., 1996). If this function is conserved in mammalian cells, the RAD23 homologue may have a direct role in cell cycle progression.
After the completion of this work, a study by Chen and co-workers was published (Withers-Ward et al., 1997) describing very similar findings. Our demonstration that HHR23A inhibits the cell cycle effect, but not the transcriptional coactivator effect, of Vpr is strong new evidence (though not a proof) in favor of human Rad23 homologue HHR23A being a component of a cell cycle regulatory pathway with which Vpr interferes.

MATERIALS AND METHODS

Plasmids

Vpr bait plasmid for the yeast two-hybrid screening was prepared by cloning of a PCR-amplified Vpr fragment from the NL4-3 molecular clone of HIV-1 into the pEG202 LexA fusion vector (Finley and Brent, 1995). Vpr plasmids for expression in human cells were constructed by cloning of PCR fragments produced from pNL4-3 or pNLA1 into pcDNA3 (Invitrogen, Carlsbad, CA). The L23A, L26A, L64A, and R80A mutants of Vpr were prepared by PCR-assisted in vitro mutagenesis of the pcDNA3-Vpr expression plasmid. HHR23A expression plasmids were prepared by insertion of the full-length HHR23A coding region (or its fragments corresponding to the yeast prey plasmids) obtained by RT±PCR from total cellular RNA in pcDNA3.

Yeast two-hybrid system

We used a version of the yeast two-hybrid system (Fields and Song, 1989; Gyuris et al., 1993) developed in R. Brent's laboratory (Finley and Brent, 1995; Gyuris et al., 1993). LexA fusion vector pEG202 was used to prepare bait plasmids. The HeLa cell acid-fusion library was used was constructed by J. Gyuris. The interaction between the DNA-binding LexA-Vpr fusion protein and target proteins was detected by transcriptional activation of two reporter genes: lacZ and LEU2. Only clones with both reporter genes activated were analyzed further. The primary selection was for cells able to form colonies in 3 to 5 days on plates without leucine. Because the prey library fusions were under the control of the GAL1 promoter and therefore were synthesized in the presence of galactose but not glucose, all putative positive clones were additionally screened for activation of β-galactosidase and for the dependence of both the ability to grow without leucine and β-galactosidase activity on the presence of galactose. Gal-dependent LEU⁺, β-gal⁺ colonies were used to rescue library plasmids. To study the specificity of the interaction, we used the interaction-mating test (Gyuris et al., 1993).

Cell transfections

Human embryonic kidney 293T cells were transfected by the calcium phosphate precipitation method as described (Felber et al., 1989). The total amount of DNA in the transfection mixtures was adjusted with Bluescript plasmid DNA to 5 μg per 60-mm plate. Human rhabdomyosarcoma A204 cells were transfected using lipofectin (Life Technologies, Gaithersburg, MD). The cells were treated with 10⁻⁷ M dexamethasone 24 h after transfection and the cell lysates were prepared after another 24 h of incubation. Luciferase activity and protein concentration were measured as described (Karl et al., 1996; Smith et al., 1996).

Flow cytometry

Two days after transfection, the cells were stained with 10 μg/ml of Hoechst 33342 (Molecular Probes, Eugene, Oregon) for 1 h at 37°C, collected, washed once with PBS, and analyzed by flow cytometry for DNA content, indicative of the phase of the cell cycle. When HIV-1 molecular clone was used for transfection, the cells were fixed with 2% paraformaldehyde prior to flow cytometry to inactivate the virus. To distinguish transfected versus untransfected cells, we included a GFP-encoding plasmid in all the transfections, allowing us to examine the DNA content per cell in green fluorescent (transfected) cells only. Use of the Hoechst 33342 dye does not require cell fixation and permeabilization interfering with GFP fluorescence.

FIG. 5. HHR23A stimulates rather than inhibits the ability of Vpr to activate glucocorticoid response. MMTV promoter-driven luciferase expression was used to measure glucocorticoid response. A204 cells were transfected with the reporter construct together with 0.6 μg of Vpr construct or Vpr and HHR23A (2 μg) constructs. Luciferase activity was measured in the absence and in the presence of dexamethasone (10⁻⁷ M).

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