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Abstract

HIV-1 reverse transcriptase is a dimeric enzyme mainly involved in the replication of the viral genome. A filamentous phage cDNA expression library from human lymphocytes was used to select cellular proteins interacting with HIV-1 reverse transcriptase. Affinity selections using the bacterially expressed monomeric large subunit of reverse transcriptase (p66) yielded host beta-actin. This clone was expressed as glutathione-S-transferase fusion protein which was identified by using a specific antibody against beta-actin. Furthermore, we show that also the eukaryotic beta-actin binds to either the large subunit of reverse transcriptase or to the Pol precursor polyprotein in vitro. The reverse transcriptase/beta-actin interaction might be important for the secretion of HIV-1 virions.
The large subunit of HIV-1 reverse transcriptase interacts with β-actin

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Received December 15, 1994; Revised and Accepted January 26, 1995

ABSTRACT

HIV-1 reverse transcriptase is a dimeric enzyme mainly involved in the replication of the viral genome. A filamentous phage cDNA expression library from human lymphocytes was used to select cellular proteins interacting with HIV-1 reverse transcriptase. Affinity selections using the bacterially expressed monomeric large subunit of reverse transcriptase (p66) yielded host β-actin. This clone was expressed as glutathione-S-transferase fusion protein which was identified by using a specific antibody against β-actin. Furthermore we show that also the eukaryotic β-actin binds to either the large subunit of reverse transcriptase or to the Pol precursor polyprotein in vitro. The reverse transcriptase/β-actin interaction might be important for the secretion of HIV-1 virions.

INTRODUCTION

The reverse transcriptase (RT) gene of HIV-1 and related retroviruses is a part of the POL open reading frame and possesses the information necessary for the replication of the viral RNA genome (for review see 1). The RT is expressed by a ribosomal frame shift during the Gag translation (2), generating a Gag-Pol precursor polyprotein (3,4). This precursor polyprotein is cleaved by the virus-encoded protease generating a number of polypeptides. One of them, a polypeptide of 66 kDa (p66) encodes the reverse transcriptase and the RNase H domain. Half of the molecules are further processed generating a 51 kDa (p51) protein with the identical N-terminus as the p66 polypeptide but lacking the C-terminus encoding the RNase H domain (3,4). In vivo both polypeptides are found in equimolar amounts forming a heterodimer (5). Dimerization is critical for the enzymatic activity (6). In the early stage of the HIV-1 life cycle the viral genome is reverse transcribed by the RT followed by integration of the resulting double-stranded DNA into the host genome as provirus. In later stages the viral particles are assembled on the cell membrane, where budding of the virus occurs. The participation of cellular proteins in the HIV-1 life cycle cannot be excluded, since a number of cellular proteins have been shown to associate with mature human immunodeficiency viruses (7). To test whether the RT interacts with cellular proteins we screened a filamentous phage cDNA expression library.

The screening of a filamentous phage cDNA expression library is principally based on the interaction cloning procedure (8,9). The library encoded polypeptides are expressed on the surface of the phages (10) containing their genetic information. Attachment of a target protein to a solid matrix allows selection and amplification of an interacting phage from the library.

We have screened a human library from peripheral lymphocytes for encoded proteins capable of binding to the large subunit (p66) of HIV-1 RT. The affinity selection yielded human β-actin. The bacterially expressed β-actin as glutathione-S-transferase (GST) fusion protein was recognized by a specific antibody, raised against the N-terminus of cytoplasmic β-actin. Furthermore the binding ability of GST-RT p66 was characterized by two different in vitro affinity binding assays using bacterially expressed GST-β-actin and native eukaryotic β-actin. The fact that native eukaryotic β-actin binds preferentially to monomeric RT p66 and to the Pol precursor polypeptide makes it possible, that this interaction is involved directly in HIV-1 virion assembly, where the Gag-Pol precursor polypeptide is also expressed as monomer. This hypothesis is supported by recent findings that assembly as well as secretion is directed into cell pseudopods organized by actin during cell-to-cell transmission (11).

MATERIALS AND METHODS

Nucleotides and other chemicals

Amersham was the supplier for the radioactivity labeled nucleotides. All other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Nucleic acids

Primers for sequencing and PCR were purchased from Microsynth (Windisch, Switzerland) and labeled as described in (12).

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Enzymes and proteins

Bovine heart muscle kinase (HMK) as well as the thrombin protease were purchased from Sigma (Buchs, Switzerland) and restriction enzymes from Boehringer-Mannheim (Rotkreuz, Switzerland).

Construction of the filamentous phage cDNA expression library

A human cDNA library has been constructed in λACT using mRNA prepared from Epstein–Barr virus (EBV) transformed human peripheral lymphocytes (13). The library contained 1.1 x 10^8 total recombinants with >95% inserts. It was amplified and converted to plasmid form (pACT) using cre-lox mediated site-specific recombination (13). The cDNA inserts from pACT (gift of C. Hovens, Zürich) were recloned into the unique BgII site of the phagemid vector pJuFo (10) based on the phagemid pComb (14). Preparation of the phages was according to (10). To test the amplification procedure, we screened the constructed expression library with protein A (Pharmacia) with an affinity to a Fc fragment that is determined to be -2 x 10^-8 M. After three rounds of selection and amplification of the phage expression library using 10 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.05% (v/v) Tween 20, all obtained clones encoded the immunoglobulin Fc fragments in frame to fos Z.

Construction of GST HIV-RT p66 fusion protein

The HIV-1 RT p66 coding sequence of pRT 100A (15) digested with HindIII and SalI was cloned into the modified expression plasmid pGEX 2T digested with SpeI and Xhol, thus generating pGEX-RT66. Both HindIII and SpeI ends were filled with Klenow DNA polymerase I before ligation. The pGEX 2T (Pharmacia) has been modified to encode a specific phosphorylation site for bovine heart muscle kinase [HMK; (9)] allowing a specific labeling of the expressed protein. Due to the cloning procedure, the GST-RT p66 fusion protein starts with the 12th amino acid HIV-1 RT p66.

Expression and purification of the GST HIV-RT p66 fusion protein

Expression of GST fusion proteins were done in E.coli strain HB101 and purification according to (16). The GST-RT had a specific enzymatic activity of 567 U/mg as measured in a standard RT assay (17).

Digestion and labeling of GST-RT p66

GST-RT p66 purified by glutathione (GSH) beads and washed with buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 2.5 mM CaCl_2 was digest with thrombin (120 U) overnight at 4°C. After centrifugation the supernatant was dialyzed against a buffer containing 20 mM Tris–HCl (pH 7.5), 25 mM NaCl and 1 mM dithiothreitol. The labeling mixture (final volume of 20 μl) contained 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 12 mM MgCl_2, 1 pmol [γ-^32P]ATP (Amersham), 1 U HMK and 1 μg of digested RT p66. The reaction was performed for 1 h at 37°C. Unincorporated [γ-^32P]ATP was separated from the labeled protein by using a G50 column (Pharmacia) equilibrated in 30 mM Tris–HCl (pH 7.5), 100 mM KCl and 0.05 % (v/v) Triton X-100.

Electrophoretic mobility shift assay

As a DNA probe we choose a partially double stranded duplex, structurally similar to a template for DNA dependent DNA synthesis. A 15mer (5'-AGTACGTCATCTCGG-3') was annealed to a 60mer (5'-CTCGAGGGTCCAGGAAATCTTGATGTTGATGTCAGATCCGAGTGACT-3') and labeled as mentioned in (12). Different amounts of protein (see Fig. 2C legend) were incubated for 30 min at room temperature with 1 pmol ^32P-labeled probe in a 20 μl reaction mixture containing 50 mM Tris–HCl (pH 7.5), 80 mM KCl, 5 mM MgCl_2 and 1 mM dithiothreitol. After incubation, glycerol was added to the reaction mixtures (10% v/v) followed by application to a non-denaturing 5% polyacrylamide gel (40:1; acrylamide:bis-acrylamide), prepared in 0.5 x TBE (18), 1% glycerol. Electrophoresis was performed at room temperature for 90 min at 150 V, after which gels were dried and subjected to autoradiography.

Screening of the library

Screening was carried out as reported by (19) with the exception that GST-RT p66 was used as target protein and GSH beads were used as solid matrix. The phages were incubated with 100 ng of GST-RT p66 for 2 h at 4°C followed by an incubation with 100 μl GSH-beads (Pharmacia) for an additional hour. The washing buffer contained 50 mM Tris–HCl (pH 7.5), 150 mM KCl and 0.05% (v/v) Triton X-100. Phages were eluted from GSH-beads by 5 mM reduced GSH dissolved in 100 mM Tris–HCl (pH 8). Host bacteria (HB101) were infected with the eluted phages according to (10), and phagemid DNA was digested with BgII in order to estimate the size of cDNA inserts and to group phagemid with identical cDNA length. Phagemid DNA isolated from the received clones was sequenced with a primer located inside the fos Z open reading frame (5'-CAAACCGAATTCGCGACGCT-3').

Polymerase chain reaction (PCR)

The PCR mixture (final volume of 20 μl) contained 10 ng of single stranded DNA of phage pool (see Fig. 3C legend) and 1 μM of primer 1 inside fos Z (5'-CAAACCGAATTCGCGACGCT-3'), 2 μM of the primer 2 inside β-actin (5'-ATCGTCGCCCCCGAAGCCCGGCCCTT-3'), 2 mM MgCl_2 and 0.5 mM each of the four dNTPs. Steps for PCR were: 94°C for 40 s, 60°C for 40 s and 73°C for 1 min. After 30 cycles the products were analyzed on a 2% low melt agarose gel.

Construction of GST-β-actin fusion protein

β-actin was excised from pJuFo clone as Xhol fragment, filled up with Klenow DNA polymerase I and cloned into the Smal site of modified pGEX 2T vector, generating pGEX-β-actin.

Expression and purification of the GST-β-actin fusion protein

Expression of the GST fusion proteins were done in E.coli strain HB101 and purified according to (16) replacing MTBS with 20
mM Tris–HCl (pH 7.5), 150 mM KCl, 1 mM dithiothreitol, 0.5 mM CaCl₂ and 0.5 mM ATP.

Expression of other recombinant proteins
GST-Abl was expressed in E. coli strain HB101 using the pGEX-Abl vector (gift of J. Wang, La Jolla, California) according to (16). Pol precursor polyprotein was expressed in E. coli strain JM109 (20). After 4 h of induction with 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG), cells were harvested, resuspended in 20 mM Tris–HCl (pH 7.5), 300 mM KCl, 1 mM dithiothreitol, 0.5 mM CaCl₂ and 0.5 mM ATP and sonicated (Branson Sonic Foxer Co., Cell disruptor B15) until 90% of the cells were broken. The sonicate was centrifuged at 15 000 g for 15 min at 4°C and lysate used in a DNAse I/β-actin affinity binding assay.

Purification of the cytoplasmic calf thymus actin
Cytoplasmic actin was purified from calf thymus according to (21) with addition of 1 mM dithiothreitol, 0.5 mM CaCl₂ and 0.5 mM ATP to all buffers.

Immunoblot analysis
GST-RT p66 and HIV-1 RT p66 were detected with a polyclonal chicken antibody against HIV-1 RT (22). GST-β-actin and calf thymus actin were detected with a polyclonal rabbit antibody specific for the N-terminus of β-actin (Ch. Chaponnier, G. Benzonana and G. Gabbiani, in preparation). Immunoblots were performed according to (23).

Glutathione affinity binding assay
Seventy μl (~2 μg) of GST-RT p66 freshly cut with thrombin and 32P-labeled (Fig. 3B, lane 1) was incubated with 30 μl DNAse beads containing 2 μg of the indicated proteins (see Fig. 4 legend) for 1 h at 4°C, followed by five washes of 1 ml each with a buffer containing 30 mM Tris–HCl (pH 7.5), 150 mM KCl and 0.05% (v/v) Triton X-100. After the fifth wash, the beads were resuspended in loading buffer and the bound protein analyzed on a 7.5% SDS–polyacrylamide gel.

DNAse I/β-actin affinity binding assay
Cytoplasmic actin was coupled to DNAse I beads according to (21) with the exception that to all buffers 1 mM dithiothreitol, 0.5 mM CaCl₂ and 0.5 mM ATP were added and that the beads were not eluted but immediately washed with 20 mM Tris–HCl (pH 7.5), 300 mM KCl, 1 mM dithiothreitol, 0.5 mM CaCl₂ and 0.5 mM ATP. Thirty μl of charged beads (containing ~2 μg actin) were incubated with 0.5 ml bacterial extract (expressing either GST-RT p66 or the Pol precursor polyprotein) for 2 h at 4°C. After incubation the beads were washed with the same buffer mentioned above. The bound fraction was separated on a 10% SDS–polyacrylamide gel, transferred to a Nylon membrane and used in an immunoblot with an antibody against HIV-1 RT p66 (22).

Other methods
Enzymatic analysis with HIV-1 RT in vitro was performed as described in (17). SDS–polyacrylamide gel electrophoresis was performed according to (24).

RESULTS
Construction of a filamentous phage cDNA display library
In order to identify possible cellular proteins interacting with HIV-1 RT we constructed a filamentous phage cDNA expression library (10) prepared from human peripheral lymphocytes infected with Epstein–Barr virus (Fig. 1A). The cDNA clones were coexpressed with the zipper domain of fos (fos Z), which was associated with the zipper domain of jun (jun Z) fused to the pIII gene product (phage coat protein; Fig. 1A and B).

Construction of the GST-RT p66 monomeric fusion protein to screen the library
We expressed the large subunit of HIV-1 RT (p66) as GST fusion protein (GST-RT p66) and could therefore first purify the fusion protein with a one-step procedure (16) and second enrich an interacting phage using GST-RT p66 charged glutathione (GSH) beads (Fig. 1C). The GST fusion protein expression vector used, contained a labeling site for bovine heart muscle kinase [HMK, (9); Fig. 2A]. Thus the GST-RT p66, purified with GSH beads, can not only be separated from the GST part and the GSH beads but can also be labeled with 32P. The purified GST-RT p66 had a molecular weight of 92 kDa, interacted with a specific chicken antibody against HIV-1 RT (Fig. 2B). A small portion of degradation products due to the expression in HB101 were also detectable. The GST-RT p66 contained a specific activity of 567
U/mg. This specific activity corresponded to 3% of the specific activity of an authentic HIV-1 RT p66 dimer (15). The reduction of the specific activity coincided with the reduced binding ability of GST-RT p66 to a double stranded DNA probe in an electrophoretic mobility shift assay (Fig. 2C). Lanes 4 and 5 show that the GST-RT p66 monomer did not bind to double stranded DNA in comparison to the authentic HIV-1 RT p66 (lanes 2 and 3, respectively). After longer exposure of the gel a weak binding could also be detected for GST-RT p66 (data not shown). Upon cleavage with thrombin the specific activity of the protein increased to 60% of the non-fused RT. Since dimerization is critical for DNA binding as well as DNA synthesis (6), we concluded that the GST-RT p66 is not able to dimerize completely. Therefore GST-RT p66 attached to GSH beads or eluted by glutathione was a monomer but appeared to be folded properly.

**Selection of the expression library with GST-RT p66 monomer yields host β-actin**

Starting from a pool of $10^{13}$ we performed four rounds of selection with GST-RT p66 as target protein (Fig. 3A). After the fourth round a clone with a cDNA insert of 2 kb length was repetitive (see Material and Methods). The partial sequence of the coding 5' end of this clone corresponded to human β-actin sequence [EMBL Data Bank: HSACTB.EM.PR, (25) and Fig. 3B]. The specificity of the selection was confirmed with PCR from DNA of phage pools received by screening the library with GST-RT p66 monomer or GST alone (control). The PCR was performed with two primers flanking the linker region of the selected β-actin plasmid (Fig. 3B). The results show that the pool received by GST-RT p66 monomer contains three independent clones, generating three different PCR products (Fig. 3C). The same products were detectable with PCR from DNA of the original phage library but to a lesser extent (Fig. 3C, lane 3). Sequencing these products revealed that the smallest product was identical to the originally obtained β-actin clone (Fig. 3C, lane 4),...
whereas the other products showed longer 5' noncoding leader-sequences of β-actin. In contrast, PCR from DNA of a phage pool received by the screening of the library with GST alone gave no PCR products (Fig. 3C, lane 1). These results suggested that the selection of the phage display library was due only to the interaction with monomeric RT p66 polypeptide.

Expression of β-actin as a GST fusion protein

To check whether the cDNA of the isolated β-actin clone contains the entire open reading frame without any deletions, it was expressed as a GST fusion protein (Fig. 3D). The obtained protein had a molecular mass of 68 kDa and interacted on an immunoblot with a specific polyclonal antibody against the N-terminal end of cytoplasmic β-actin, indicating that the amplified β-actin clone expressed the whole β-actin open reading frame. The isolated GST-β-actin was copurified with degradation products (due to expression in HB (101) and bacterial chaperonin GroEL (identified by immunoblot, data not shown), suggesting an incomplete native conformation of the fusion protein, as has been shown in eukaryotes (26). Therefore, we took into account this unavoidable copurification when performing further interaction studies. The use of 0.5 mM CaCl_2 and 0.5 mM ATP in order to maintain the native structure of β-actin (27), prevented a detection of the influence of β-actin on RT p66 (either as authentic or fusion protein) in a RT assay under standard conditions (17). But since the GST-RT p66 used for phage screening was unable to dimerize (see Fig. 2C) we presumed that β-actin is bound preferentially to RT p66 as monomer.

GST-β-actin interacts with the processed GST-RT p66

To characterize the interaction of HIV-1 RT to β-actin in vitro, we used GST-β-actin beads and GST-RT p66 additionally digested and labeled with 32P (see Materials and Methods; Fig. 4, lane 1). The removal of the GST part by thrombin was necessary to avoid binding of the GST-RT p66 to GSH-beads without interacting with β-actin. The same buffer conditions were used for the library screening (see Fig. 3A) were applied in this characterization. Quantification of the bound cut RT p66 revealed a 6-fold increase of binding of β-actin in comparison to the controls being either the GST alone (Fig. 4, lane 2) or the GST-Abl fusion protein (Fig. 4, lane 3). This was the highest observed level of interaction, explainable by partially unfolded GST-β-actin fusion protein (containing the chaperonin GroEL) as well as the fact that GST-RT p66 after digestion, starts to form an active homodimer. Binding of RT p66 to β-actin was stable even after the addition of 500 mM KCl or 2% (v/v) Triton X-100 (data not shown). Thus, the interaction found by screening the library could be reproduced by using the in vitro system described here.

Binding of GST-RT p66 and Pol Precursor to eukaryotic actin

To overcome the GroEL copurification with bacterial GST-β-actin we immobilized calf thymus actin (98% homology to human actin; (25)) on DNAse I coupled beads (21). Figure 5A (lane 2) shows the bound actin fraction after extensive washing of the DNAse I beads. The presence of β-actin inside this bound fraction was determined by immunoblot using a β-actin specific antibody (Fig. 5A, lane 3). Using these DNAse I/β-actin beads we tested the binding ability of GST-RT p66 to β-actin. Figure 5B shows the binding of GST-RT p66 to the DNAse I/β-actin beads in the presence of 300 mM KCl. No binding could be detected using DNAse I beads lacking β-actin (negative control, Fig. 5B, lane 1). To test whether the RT as Pol precursor polypeptide also interacts with β-actin we repeated the same experiment with the Pol precursor polypeptide instead of GST-RT p66. Figure 5C (lanes 2) shows the binding of Pol precursor polypeptide to the DNAse I/β-actin beads. As with GST-RT p66 no binding could be detected using DNAse I beads lacking β-actin (Fig. 5C, lane 1). The same controls used in Figure 4 (GST alone: lane 2, GST-Abl fusion protein: lane 3) did not show any binding (data not shown) suggesting that the binding of the GST-RT p66 and Pol precursor polypeptide were specific.

Taken together these findings indicated that the biological function of the interaction could play a critical role in later stages (assembly and budding) of the HIV-1 life cycle.
DISCUSSION

Screening a filamentous phage cDNA expression library from human lymphocytes we found an interaction between the large subunit of RT (p66) and human β-actin. This interaction could be reproduced with two different in vitro affinity binding systems using bacterially expressed and eukaryotic β-actin.

Actin was originally identified in the cytoplasmic compartment of cells as part of the cytoskeleton and many different isoforms of actin exist in higher eukaryotes. The significance of these different isoforms appears to be a functional diversity. Isoforms are differentially expressed in different specialized tissue cells and within a single cell there may exist different isoforms which segregate to a different region of the cell (25). In particular, it has been shown that the β-actin isoform is located at the plasma membrane and the regions of moving cytoplasm (28).

The influence of viral or cellular molecules mediating the proper assembly of the virions are still elusive. During the formation of virions the Gag and Gag–Pol precursor polyproteins are myristylated at their N-termini and located at the inner plasma membrane of the host cell (29). It was shown for HIV-1 and other retroviruses that in an infected cell the mentioned precursor polyproteins can be concentrated unidirectionally to the tip of a cell pseudopod which is formed by an unidentified actin-isoform (11,30–32). Additionally an unidentified isoform of actin has been found in HIV-1 (7) and a number of other retroviruses (33–37).

Our results suggest that actin interacting with RT might be responsible for an induced membrane translocation of the viral Gag–Pol precursor polyprotein to the tip of the pseudopod for cell-to-cell transmission, a crucial step for the assembly and further secretion of HIV-1. Thus, RT may not only play an important role in the replication of the viral genome, but also in the production of mature virions (38).

ACKNOWLEDGEMENTS

We thank Marc Suter (Zürich) for instruction in using the filamentous phage expression system, Chris Hovens (Zürich) for donating the human cDNA library (pACT vector) and John Silke for critical reading of the manuscript. This work was supported by the Swiss National Science Foundation (AIDS program 31-37146.93). MH is a recipient of the Swiss National Science Foundation Grant for the MD-PhD program (3135-36713.92).

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