Analysis of cross reactivity of retrovirus proteases using a vaccinia virus–T7 RNA polymerase-based expression system

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We have used the vaccinia virus–T7 RNA polymerase-based expression system for studies on the activity of proteases from various retroviruses on homologous and heterologous Gag polyproteins in eukaryotic cells. Proteases from human immunodeficiency virus (HIV) types 1 and 2, equine infectious anaemia virus, human T cell leukaemia virus type 1 and human spumavirus were produced and were shown to cleave their cognate Gag substrates produced in trans. Analysis of cross reactivity revealed that lentivirus proteases cleaved only lentivirus Gag proteins and oncovirus proteases acted primarily on oncovirus Gag proteins. The HIV-2 protease cleaved the HIV-1 Gag precursor almost as efficiently as HIV-1 protease. Expression of the 5’ end of the human spumavirus pol gene revealed that it encodes a functional protease that acts specifically on the human spumavirus Gag polyprotein. This assay will allow further investigation on the activity and specificity of retrovirus proteases in eukaryotic cells.

Introduction

The family Retroviridae can be divided into the three subfamilies lentiviruses, oncoviruses and spumaviruses. The gag and gag–pol genes of all retroviruses are expressed as polyprotein precursors. The Gag polyprotein is subsequently processed to the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins, and in some cases into additional Gag proteins with uncertain functions (Weiss et al., 1985; Wills & Craven, 1991). The Gag–Pol precursor contains, in addition to Gag, the enzymes protease (PR), reverse transcriptase (RT)/RNaseH and integrase (IN) (Gelderblom, 1991; Weiss et al., 1985; Wills & Craven, 1991). In lentiviruses, PR is encoded by the pol gene (Kräusslich & Wimmer, 1988; Oroszlan & Luftig, 1990). The human T cell leukaemia virus type 1 (HTLV-1) subfamily of oncoviruses produce PR from a separate reading frame located between gag and pol and in other oncoviruses the PR is encoded by either the gag or the pol gene (Kräusslich & Wimmer, 1988; Oroszlan & Luftig, 1990). The human spumavirus (HSRV) PR appears to be situated at the N terminus of Pol (Aguzzi, 1993).

Retrovirus PRs belong to the family of aspartic proteases and many retrovirus PRs exhibit strong similarity to a consensus region (LVDTGA) that is present in the active site of cellular aspartic proteases. Mutagenesis of the human immunodeficiency virus type 1 (HIV-1) PR has confirmed the functional importance of the aspartic acid and other amino acids in this region of PR (Loeb et al., 1989; Seelmeier et al., 1988). A single point mutation in the active site of the HIV-1 PR abrogates the maturation process and renders the virions non-infectious (Göttlinger et al., 1989; Mergener et al., 1992).

The capsids of type B and D oncoviruses and spumaviruses assemble in the cytoplasm, after which they are transported to the cell membrane where budding occurs (Gelderblom, 1991; Weiss et al., 1985). In contrast, in the HTLV-1 family, the C type oncoviruses and the lentiviruses, the Gag and Gag–Pol precursors are transported to the plasma membrane where the virions assemble concomitantly with budding (Gelderblom, 1991; Weiss et al., 1985). At the time of virus release, PR cleaves the Gag and Gag–Pol polyproteins which results in virus maturation (Gelderblom, 1991; Weiss et al., 1985; Wills & Craven, 1991). Overexpression of HIV-1 PR in virus-producing cells results in premature cleavage of Gag and a reduction of virus infectivity (Luukkonen et al., 1995). A role for HIV-1 PR and equine infectious anaemia virus (EIAV) PR in the early phase of the replicative cycle, immediately after penetration of the cell, has been proposed (Nagy et al., 1994; Roberts et al., 1991). How PR activity is regulated during the virus life cycle remains unclear.
Retrovirus Gag and Gag-Pol precursors contain a number of cleavage sites for PR, but the primary amino acid sequence at each site is unique (Kräusslich & Wimmer, 1988; Oroszlan & Luftig, 1990). Since even the two amino acids surrounding the scissile bond vary between different sites, the exact cleavage specificities of retrovirus PRs have remained elusive (Pearl & Taylor, 1987). In an extensive study using in vitro translated HIV-1 p55Gag polyproteins, Trich and coworkers demonstrated that amino acids other than those to which HIV-1 PR binds directly are important in determining the cleavage rate of a site (Trich et al., 1991). This implies that structure as well as primary sequence may be crucial for cleavage site recognition and utilization. Others have shown that individual PR substrates act independently in recognizing and accommodating amino acids other than those to which HIV-1 PR binds (Cameron et al., 1994). The combination of amino acids present in the substrate binding pocket appears to determine cleavage specificity. Interactions between the PR substrate binding pocket and the substrate appear to be mediated primarily by van der Waal’s forces (Cameron et al., 1994). This arrangement may explain how the retrovirus PR can recognize substrates with apparent differences in amino acid primary sequence.

The strict regulation of PR activity in the viral life cycle emphasises the importance of analysing PR activity on its natural substrate. In the present study we have used the vaccinia virus–T7 RNA polymerase-based expression system (Fuerst et al., 1986, 1987) for studies on activity and specificity of retrovirus PRs in eukaryotic cells. Our results demonstrate that retrovirus PRs expressed in this system are enzymatically active and cleave their homologous Gag substrates. Lentivirus PRs generally act only on other lentivirus Gag proteins, and oncovirus PRs act primarily on oncovirus Gag proteins. Expression of the putative human spumavirus PR revealed that it is located towards the N terminus of Pol and acts specifically on the HSRV Gag polyprotein. This assay will allow us to further investigate the activity and specificity of retroviral PRs in eukaryotic cells, and may be a useful tool for studies on antiviral compounds directed against PR.

Methods

Plasmid constructs. To construct plasmids containing the PR coding sequence from various retroviruses under control of the bacteriophage T7 promoter, the PR coding sequences were PCR-amplified and cloned into an EcoRV-digested, calf intestinal alkaline phosphatase (CIAP)-treated pBluescript plasmid (Stratagene). The HIV-2 PR coding sequence was PCR-amplified from the HIV-2 molecular clone HIV-2ISY (Franchini et al., 1989) using oligonucleotides HIV2PR-START (5’ TTGCGCGCGAAATGCCTCAGATCTTCCTTTGGG 3’) introducing a translational start codon, and HIV2PRSTOP (5’ GTTACTATAATTTAATGACATG 3’) introducing two stop codons. The EIAV PR was PCR-amplified from a genomic EIAV clone (Stephens et al., 1986), kindly provided by N. Rice, using oligonucleotides EIAVPRSTART (5’ TTGGCGCGCGAAATGGTAACATCTATGTCATC 3’) and EIAVPRSTOP (5’ TTGACTATTAACCTCTCTTTGG 3’) introducing two stop codons. The EIAV PR was PCR-amplified from plasmid pHSVGAG (Pol kindly provided by G. N. Pavlakis), containing human spumavirus gag and pol sequences (Flügel et al., 1987; Maurer et al., 1988), using oligonucleotides HSRVPRSTART (5’ TTGCGCGCGAAATGCCAGTTATACCGTTAGATY) and HSRVPRSTOP (5’ GCCAGTTATACCGTTAGATY) that introduces a start codon, and PRSTOP (5’ AAATTTAATTAAAATGTTGAG 3’) introducing two stop codons. The pT7-PRM plasmid was generated by PCR amplification of the PR-coding sequence of pSVGAGPOL-RRE-PolyA (Kräusslich et al., 1991). This plasmid was constructed by first PCR amplifying the Gag-PR region of pSVGAGPOL (pT7Gag/MA and pSVGAG/CA) coding sequence pT7-HIV1Gag was constructed by PCR-amplification of the gag coding sequence of pHXB2 (Ratner et al., 1987a, b) using oligonucleotides BSS (5’ GGCTTGCAGAGCGAGCAGGGCAGCAGGAG 3’) and 7492 (5’ TTATATGCAAGGAGTGTGAG 3’). The PCR fragment was blunt-end ligated into an EcoRV-digested, CIAP-treated pBluescript generating pT7-HIV1Gag. The pT7-GagPR plasmid was constructed by first PCR amplifying the Gag –PR region of pHXB2 (Ratner et al., 1987a, b) using oligonucleotides BSS and PRSTOP, followed by blunt-end cloning into an EcoRV-digested, CIAP-treated pBluescript. The infectious molecular clone of HIV-1, pNL43 (kindly provided by M. Martin) has been described before (Adachi et al., 1986). Plasmid pH7T7IV2Gag was constructed by PCR amplification of the HIV-2 Gag coding sequence from the molecular clone HIV-2 ISY (Franchini et al., 1989), using oligonucleotides 7105 (5’ CATGCGCGCAGATTGGGGAGTGGCG 3’) and 7422 (5’ CAGAAAACCTTTGCTGGTGCATC 3’), followed by cloning into pBluescript. Plasmid pT7-HTLV1Vag was generated by subcloning of the gag-containing Sall/Asp718 fragment of pNL-HTLVag, kindly provided by G. N. Pavlakis, into SalI/Asp718-digested pBluescript. To construct pT7-7BLVag, the Gag-coding sequence was PCR amplified from the genomic molecular clone of bovine leukemia virus (BLV), P913-CP (Dersch, 1987; Felber et al., 1989) kindly provided by D. Derse) followed by cloning into pBluescript. Plasmid pT7-7BLVag was constructed by subcloning the gag-containing HindIII/Asp718 fragment of pNL7BLVag into HindIII/Asp718-digested pBluescript. To construct pT7-7EIAVag, the Gag-coding sequence was PCR amplified from the genomic molecular clone of the recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (vTF7-3) (Fuerst et
with 10% non-fat dried milk, the filters were washed three times in PBS-Tween and developed with enhanced chemiluminescence (ECL) detection reagents (Amersharm). Filters were washed three times as described above, and incubated with horse-radish peroxidase (HRP)-labelled secondary antibody for 1 h. The filters were washed three times as described above, and incubated with horse-radish peroxidase (HRP) -labelled secondary antibody for 1 h. The filters were washed three times in PBS-Tween and incubated with primary antibody for 1 h. The filters were washed three times as described above, and incubated with horse-radish peroxidase (HRP) -labelled secondary antibody for 1 h. The filters were washed three times in PBS-Tween supplemented with 10% non-fat dry milk.

Antisera. Primary antibody for HIV-1 Gag detection was a mouse monoclonal antibody against HIV-1 p24Gag (EF7) (Hinkula et al., 1990), kindly provided by J. Hinkula and B. Wahren, used at a dilution of 1:7500. HIV-2 Gag was detected with serum from an HIV-2-infected individual (kindly provided by R. Thorstensson), diluted 1:1000. EIAV Gag was detected using a rabbit polyclonal serum against EIAV p26Gag (Henderson et al., 1987) (kindly provided by S. Oroszlan), diluted 1:5000. HTLV-1 Gag was detected using an HTLV-1 positive human serum (Scripps), diluted 1:100. BLV Gag was detected using serum from a BLV-infected sheep (kindly provided by D. Derse) at a dilution of 1:500. HSRV Gag was detected using a rabbit polyclonal serum against the major capsid protein of HSRV (Aguzzi et al., 1993; Baunach et al., 1993) (a kind gift of A. Rethwilm), diluted 1:1000. Secondary anti-mouse antibody (Amersham) was diluted 1:20000, anti-rabbit antibody (Amersham) 1:1000, anti-human antibody (Amersham) 1:10000, and anti-goat antibody (Dakopatts), used for detection of sheep antibody, was diluted 1:500. All antisera were diluted in PBS-Tween supplemented with 10% non-fat dry milk.

Results

Coexpression of HIV-1 PR and HIV-1 p55Gag using the vaccinia virus–T7 expression system results in specific cleavage of p55Gag

To investigate whether the vaccinia virus–T7 RNA polymerase-based expression system could be used to study activity of retrovirus proteases, we first constructed plasmid pT7-GagPR (Fig. 1) which contains the HIV-1 p55Gag and PR coding sequence under control of the bacteriophage T7 RNA polymerase promoter. Transcription from the T7 promoter can be activated by transfection of HeLa cells infected with the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986, 1987) producing T7 RNA polymerase. Production of p55Gag polyprotein was analysed by Western immunoblotting using a mouse monoclonal antibody directed against HIV-1 p24Gag (Hinkula et al., 1990). High levels of unprocessed p55Gag were produced (Fig. 2a). In addition, the cleavage product p41Gag and low levels of the mature p24Gag protein were detected. In contrast, plasmid pT7-HIV1Gag (Fig. 1) which contains the p55Gag coding sequence but not the PR coding sequence, produced only p55Gag (Fig. 2a). These results demonstrated that p55Gag was specifically cleaved by the HIV-1 PR in this expression system.

Although cleavage of p55Gag was observed, a large fraction of p55Gag remained unprocessed. Since the ribosomal frameshift signal in Gag was intact in plasmid pT7-GagPR, only about 5 to 10% of all p55Gag molecules were predicted to contain also PR (Hatfield & Oroszlan, 1990; Jacks et al., 1988). The levels of this p55Gag-PR fusion protein were below detection level. In an effort to improve cleavage efficiency, a plasmid designed to produce the HIV-1 PR was generated by PCR amplification of the HIV-1 PR coding sequence from the infectious HIV-1 molecular clone pHXB2 (Ratner et al., 1987a, b). The PCR-amplified PR coding sequence was provided with translation start and stop
HIV1PR. Fig. 2(a) shows that cleavage of p55Gag was observed only in the presence of pT7-HIV1PR, demonstrating that HIV-1 PR produced in trans can cleave its p55Gag substrate. Transfection with pT7-PRM (Fig. 1) that produces a negative PR mutant in which the Asp-25 residue was changed to Gly (Smith et al., 1993), did not give rise to processing of the p55Gag substrate (Fig. 2c). In an effort to estimate expression levels of HIV-1 PR in the transfected cells, a Western blot was performed using a PR-specific serum. However, PR could not be detected (data not shown).

We next wished to compare the cleavage products obtained from pT7-HIV1Gag in the presence of PR with those obtained in cells expressing the entire HIV-1 genome. HeLa cells were therefore transfected with the infectious HIV-1 proviral clone pNL43 (Adachi et al., 1986) and cell extract was prepared and subjected to immunoblotting. The p41Gag protein was present also in cells transfected with the infectious proviral clone pNL43 (Fig. 2b). The p41Gag protein consisted of p17Gag and p24Gag, as evidenced by comigration with p41Gag produced from pT7-41Gag (Fig. 2b), designed to produce p41Gag consisting of p17Gag and p24Gag (Schwartz et al., 1992) (Fig. 1). These results demonstrated that the cleavage site located after p24Gag is efficiently processed compared to the site located between p17Gag and p24Gag.

The cell culture medium of cultures transfected with pNL43 or pT7-HIV1Gag were analysed for the presence of particle-associated, polyethylene glycol-precipitable HIV-1 Gag proteins. While high levels of virus were produced from pNL43-transfected cells, only low levels of extracellular Gag were found in cell cultures transfected with pT7-HIV1Gag (data not shown).

Cleavage efficiency is dependent on efficient PR dimerization, and on Gag and PR concentrations

To investigate the sensitivity of the PR assay, cells infected with T7 RNA polymerase-producing vaccinia virus were transfected with serially diluted pT7-HIV1PR and pT7-HIV1Gag. The results showed that PR was active over a wide range of concentrations (Fig. 3). We observed a proteolytic effect using as little as 10 ng PR plasmid, demonstrating that low levels of PR could be detected. However, a significant amount of p55Gag remained uncleaved. Since PR dimerization is required for enzymatic activity, dimerization may be a rate-limiting step for processing of p55Gag. To investigate whether cleavage efficiency could be improved using a genetically linked, one chain PR dimer (2PR) (Burstein et al., 1991; Kräusslich, 1991), we cotransfected pT7-2PR (Kräusslich, 1991) (Fig. 1) with pT7-HIV1Gag. The 2PR was found to be active at a concentration approximately
Fig. 3. The HIV-1 PR expressed in trans is active over a wide range of concentrations. HeLa cells infected with recombinant vaccinia virus vTF7-3 producing bacteriophage T7 RNA polymerase were cotransfected with plasmid pT7-HIVGag (4 μg) producing the HIV-1 p55Gag precursor, and 0.01 μg, 0.1 μg or 1 μg of plasmid pT7-HIV1PR expressing a monomeric HIV-1 PR or 0.01 μg, 0.1 μg, or 1 μg of pT7-2PR producing a genetically linked PR dimer (Kräusslich, 1991). Cell extracts were analysed by Western immunoblotting using a mouse monoclonal antibody against HIV-1 p24Gag (Hinkula et al., 1990). The unprocessed p55Gag precursor and the cleavage products p41Gag and p24Gag are indicated on the right.

Fig. 4. HeLa cells infected with recombinant vaccinia virus vTF7-3 producing bacteriophage T7 RNA polymerase were transfected with 4 μg, 2 μg, 1 μg or 0.5 μg of the HIV-1 p55Gag-producing plasmid pT7-HIVGag in the absence or presence of 0.5 μg of HIV-1 PR-expressing plasmid pT7-HIV1PR. Cell extracts were analysed by Western immunoblotting using a mouse monoclonal antibody against p24Gag (Hinkula et al., 1990). The unprocessed p55Gag precursor and the cleavage products p41Gag and p24Gag are indicated on the right.

10-fold lower than the monomeric PR (Fig. 3, compare lanes 2 and 6). When using high amounts of pT7-2PR, a general decrease of Gag was observed (Fig. 3). Similar results were obtained when transfecting high amounts of pT7-HIV1PR (see Fig. 7). This may be a result of cytotoxic effects of the PR (Kräusslich, 1991).

Alternatively, the reason for the inability of PR to process p55Gag efficiently may be caused by different localization of PR and p55Gag within the cell. Since PR expressed here does not contain the myristoylation signal normally present in the Gag-Pol precursor, PR may remain in the cytosol, while the Gag substrate is transported to the plasma membrane after translation. We therefore investigated whether the concentration of p55Gag protein could influence the levels of cleavage products. Different amounts of pT7-HIVGag were transfected while the amount of pT7-HIV1PR was kept constant. When low amounts of plasmid pT7-HIVGag were used, cleavage products could not be detected. The higher amount of substrate plasmid transfected, the more of the cleavage intermediate p41Gag appeared (Fig. 4). The reason for this may be that the subcellular compartment in which the Gag proteins reside may be saturated at high Gag concentrations, thereby increasing the pool of Gag molecules accessible to PR.

Taken together, these results indicated that our assay for PR activity is sensitive, and detects low levels of PR in a dose-dependent manner. The requirement for PR homodimerization and the accessibility of Gag to PR appear to limit the efficiency of this system.

HIV-2, EIAV and HTLV-1 PR produced in the vaccinia virus-T7 system efficiently cleave their cognate Gag substrates

We next wished to investigate whether other retroviral PRs could be expressed in the vaccinia virus-T7 expression system. The PR coding sequences from two lentiviruses (HIV-2 and EIAV) and one oncovirus (HTLV-1) were PCR amplified and cloned downstream of the T7-promoter as described in the Methods. Similarly, HIV-2, EIAV and HTLV-1 gag sequences were PCR amplified and cloned downstream of the T7-promoter. Production of PR or Gag from these plasmids
was analysed after transfection into HeLa cells infected with the recombinant vaccinia virus producing T7 RNA polymerase (Fuerst et al., 1986, 1987).

Expression of HIV-2 Gag in the absence of PR gave rise to two proteins (Fig. 5a). These proteins corresponded in size to the p58Gag precursor and a cleavage intermediate with a predicted molecular mass of 43 kDa, presumably consisting of p16Gag/MA and p28Gag/CA. Plasmid pT7-HIV2Gag was cotransfected with a serial dilution of the HIV-2 PR producing plasmid, pT7-HIV2PR. When 1 µg pT7-HIV2PR was included in the transfection, the amount of p58Gag decreased, and two faster migrating bands appeared (Fig. 5a). One band corresponded to the predicted cleavage product p28Gag/CA. The other band migrated at around 33 kDa, and may correspond to a cleavage intermediate consisting of p28Gag/CA and p8Gag/NC, with a predicted molecular mass of 33 kDa. Lower PR concentrations had no detectable effect on the Gag precursor. This is in contrast to the results obtained with HIV-1 PR, where as little as 10 ng PR plasmid resulted in specific cleavage of HIV-1 p55Gag.

Similarly to HIV-2 Gag, the EIAV p55Gag polyprotein was cleaved in the absence of PR. This cleavage gave rise to a protein corresponding in size to a previously described 49 kDa protein (Fig. 5b) (Henderson et al., 1987). Additional cleavage and the appearance of 40 kDa and 26 kDa cleavage products was observed only in the presence of the EIAV PR expression plasmid pT7-EIAVPR (Fig. 5b). EIAV Gag cleavage products with molecular masses of 49 kDa, 40 kDa and 26 kDa have been described before (Henderson et al., 1987). Transfection of pT7-EIAVGag with serially diluted pT7-EIAVPR showed that EIAV PR-dependent cleavage is detected only when high amounts of pT7-EIAVPR (5 µg) were used (Fig. 5b), thus suggesting that EIAV PR acts less efficiently on EIAV Gag than HIV-1 and -2 PRs on their cognate Gag proteins.

In contrast to HIV-2 and EIAV Gag, the HTLV-1 Gag expression plasmid pT7-HTLV1Gag produced only one protein corresponding in size to the previously described p53Gag precursor (Miyakoshi et al., 1992) (Fig. 5c). Cotransfecting pT7-HTLV1PR and pT7-HTLV1Gag gave rise to cleavage products migrating as 40 kDa and 24 kDa (Fig. 5c). These proteins were also detected using a monoclonal antibody against HTLV-1 p24Gag/CA (Miyakoshi et al., 1992), kindly provided by H. Miyakoshi (data not shown). The 40 kDa protein may correspond to a cleavage intermediate consisting of p19Gag/MA and p24Gag/CA with a predicted molecular mass of 38 kDa. The 53 kDa, 40 kDa and 24 kDa proteins were also present in the HTLV-1-infected MT-2 cell line (Miyoshi et al., 1981) (Fig. 5c). Cleavage was detected using low amounts of pT7-HTLV1PR plasmid (0.1 µg). Furthermore, in the presence of high levels of pT7-HTLV1PR, the majority of p53Gag was cleaved.

Taken together, these results demonstrated that PRs from several retroviruses cleave their cognate Gag substrates when produced in trans in the vaccinia virus
T7 RNA polymerase-based expression system. The amount of PR-expressing plasmid required for cleavage to occur varied between the various PRs. The reason for this may be that PRs from different retroviruses work with different efficiencies in the intracellular environment. Alternatively, these differences may reflect differences in PR stability or accessibility of various Gag proteins to PR within the cell.

The 5' end of the HRSV pol gene encodes a functional protease

Based on sequence comparison, the HRSV PR appears to be encoded within the pol coding sequence (Aguzzi, 1993; Fligel, 1991; Maurer et al., 1988). Recently it was shown that the N terminus of Pol was cleaved to yield a 10 kDa protein in infected cells (Netzer et al., 1993). To confirm that this indeed corresponded to PR, the 5' end of HRSV pol was PCR amplified and cloned downstream of the T7 promoter, resulting in pT7-HSRVPR. This plasmid was cotransfected with pT7-HSRVGag, designed to produce the HSRV Gag polyprotein. Gag production was assessed by Western immunoblotting using a rabbit polyclonal antiserum against HSRV CA protein (Aguzzi et al., 1993; Baunach et al., 1993). In the absence of PR, pT7-HSRVGag produced a single protein corresponding in size to the predicted 72 kDa Gag precursor (Fig. 6). Cotransfections of high amounts (5 µg) of PR plasmid and pT7-HSRVGag, gave rise to a cleavage product corresponding in size to the previously described p32Gag/CA protein (Fig. 6) (Baunach et al., 1993). Transfections with lower amounts of PR plasmid did not give rise to significant Gag cleavage. The experiments demonstrated that the HSRV PR is situated at the N terminus of Pol.

The HIV-2 PR efficiently cleaves the HIV-1 p55Gag polyprotein

The sequence identity between HIV-1 and HIV-2 PR is approximately 50% (Myers, 1990) and cleavage of the HIV-1 Gag precursor by the HIV-2 PR in vitro has been described (Le Grice et al., 1989; Wu et al., 1990). To test if the HIV-2 PR could cleave HIV-1 p55Gag in this expression system, we cotransfected different concentrations of pT7-HIV1PR or pT7-HIV2PR with pT7-HIV1Gag. When using 0.1 µg of PR plasmid, only the HIV-1 PR cleaved out the HIV-1 p41Gag intermediate (Fig. 7), whereas at higher concentrations of PR-expressing plasmids, HIV-1 and HIV-2 PRs worked with equal efficiency (Fig. 7). In addition to p41Gag the HIV-2 PR appeared to cleave out a protein migrating slightly faster than p41Gag, indicated in the figure as p39Gag (Fig. 7). This protein may consist of p24Gag and p15Gag, or may be a truncated form of the p41Gag protein. The latter explanation is unlikely since the p24Gag monoclonal antibody used here binds to the extreme C terminus of p24Gag. In conclusion, our results showed that the HIV-2 PR cleaves the HIV-1 p55Gag polyprotein in eukaryotic cells.
Cross activity of PRs from different subfamilies

To investigate the cross activity between the retroviral PRs expressed in this system, the activity of different PRs on Gag proteins from various retroviruses were analysed. Specific cleavage was determined by Western immunoblotting. First we wished to investigate activity of PRs from different subfamilies on lentiviral Gag polyproteins. The HIV-1 p55Gag precursor was specifically cleaved by the HIV-2 PR, as shown in Fig. 7. Also the EIAV PR cleaved the p55Gag protein to generate the HIV-1 p41Gag intermediate, albeit at low efficiency (Fig. 8a). However, both the HTLV-1 PR and the HSRV PR failed to process the HIV-1 p55Gag precursor (Fig. 8a). We were unable to detect cleavage of HIV-2 Gag by heterologous PRs (results not shown). On the other hand, the EIAV p55Gag precursor was processed by HIV-1 PR and HIV-2 PR (Fig. 8b). Interestingly, low levels of processing were also observed with the HTLV-1 PR (Fig. 8b). These results showed that lentivirus Gag polyproteins were cleaved primarily by lentivirus PRs.

Next we investigated the ability of various PRs to act on oncovirus Gag precursors. Cleavage of the HTLV-1 p53Gag precursor by heterologous PRs could not be detected (data not shown). We therefore generated plasmid pT7-BLVGag encoding the BLV gag gene. Plasmid pT7-BLVGag generated a protein corresponding in size to the previously described p46Gag precursor (Oroszlan & Luftig, 1990) (Fig. 8c). This precursor was cleaved by the HTLV-1 PR (Fig. 8c). The observed cleavage product corresponded in size to a Gag intermediate with a predicted molecular mass of 37 kDa, presumably containing p15Gag/MA and p24Gag/CA. Cleavage of BLV Gag was not observed in the presence of the lentivirus EIAV PR or the HSRV PR (Fig. 8c). We also tested if the various PRs could cleave the HSRV Gag precursor. Cotransfection experiments revealed that the Gag precursor was not cleaved by oncovirus or lentivirus PRs (data not shown).

PRs from different retroviruses may be able to dimerize and form inactive heterodimers. Since HTLV-1 PR and HSRV PR did not cleave the HIV-1 p55Gag protein, we tested the ability of HIV-1 PR to process HIV-1 p55Gag in the presence of excess HTLV-1 PR or HSRV PR. One μg of pT7-HIV1PR was cotransfected with 5 μg pT7-EIAVPR or 5 μg of pT7-HSRVPR in the presence of pT7-HIV1Gag. These PRs did not negatively affect HIV-1 PR-mediated p55Gag processing (Fig. 9).

Discussion

Here we have used the vaccinia virus T7 RNA polymerase-based eukaryotic expression system (Fuerst et al., 1986, 1987) to express retroviral PRs in eukaryotic cells. The PRs of five different retroviruses were successfully produced and were shown to specifically cleave their cognate Gag substrates. Although PRs of HIV-1, HIV-2, EIAV and HTLV-1 produced here in eukaryotic cells also have been expressed in bacteria, the HSRV PR has not been identified before. We showed that the N terminus of HSRV Pol encodes a PR which specifically cleaves HSRV Gag. Analysis of cross activity revealed that HIV-2 and EIAV PRs also cleaved the HIV-1 Gag precursor and that HIV-1 and HIV-2 PRs were able to process EIAV Gag. HTLV-1 PR cleaved only HTLV-1 Gag and BLV Gag. HSRV failed to cleave lentivirus or oncovirus Gag proteins and HSRV Gag was not processed by lentivirus or oncovirus PRs. Interestingly, the specificity of retrovirus PRs can be extended to several species within the subfamily. However, cleavage of Gag proteins from other retrovirus subfamilies did generally not occur.

Detailed analysis of HIV-1 Gag processing showed that expression of PR and Gag substrate in trans resulted in cleavage of Gag and generation of cleavage products similar in size to those found in cells transfected with an infectious molecular clone of HIV-1. The HIV-1 PR provided in trans efficiently cleaved out a Gag intermediate migrating as a 41 kDa protein. Plasmid pT7-41Gag, designed to produce a Gag protein consisting of p17Gag/MA and p24Gag/CA, produced a protein migrating at the same position as the p41Gag protein generated from p55Gag in the presence of PR. However, little mature p24Gag was detected in the presence of PR. From these data, we concluded that the proteolytic cleavage sites located immediately after p24Gag are utilized more efficiently than the site between p17Gag and p24Gag. These results are in agreement with in vitro results on cleavage efficiencies for different sites within the p55Gag polyprotein. Trich et al. reported that the first cleavage in p55Gag occurred at the p2/p7 boundary, located close to the C terminus of p24Gag, followed by cleavage at the p7/p6 boundary. The third site to be processed was the site between p17Gag and p25Gag, and the last processing event separated p25Gag from p2Gag (Trich et al., 1991).

Proteolytic processing of HIV-1 Gag could be seen using as little as 10 ng of pT7-HIV1PR, demonstrating that PR is highly active within the cell. In spite of this fact, a large proportion of HIV-1 p55Gag remained uncleaved in the presence of PR. The reason for this may be that PR and Gag do not co-localize within the cell. In addition to subcellular localization of PR, dimerization efficiency may be an important determinant of PR activity within the cell. A comparison in activity between PR expressed from pT7-HIV1PR and pT7-2PR revealed that the genetically linked dimer functions approximately 10-fold more efficiently than the monomer. A previous study showed a two- to fivefold higher activity of the
Retrovirus proteases

(a) HIV-1 Gag

(b) EIAV Gag

(c) BLV Gag

Fig. 8. Activity of retrovirus proteases on different Gag polyproteins. (a) HeLa cells infected with recombinant vaccinia virus vTF7-3 producing bacteriophage T7 RNA polymerase were cotransfected with the HIV-1 p55Gag-expressing plasmid pT7-HIV1Gag (4 µg) and 5 µg of pT7-EIAVPR, pT7-HTLV1PR or pT7-HSRVPR, respectively. Cell lysates were analysed by Western immunoblotting using a mouse monoclonal antibody against HIV-1 p24Gag (Hinkula et al., 1990). Unprocessed p55Gag and the p41Gag cleavage product are indicated on the right. (b) Vaccinia virus vTF7-3-infected HeLa cells were cotransfected with the EIAV p55Gag-expressing plasmid pT7-EIAVGag (4 µg) and 5 µg each of pT7-HIV1PR, pT7-HTLV1PR, pT7-EIAVPR, pT7-HTLV1PR or pT7-HSRVPR, respectively. Cell lysates were analysed by Western immunoblotting using a rabbit polyclonal serum against EIAV p26Gag (Henderson et al., 1987). Unprocessed p55Gag and the p41Gag cleavage product are indicated on the right. (c) Vaccinia virus vTF7-3-infected HeLa cells were cotransfected with the BLV Gag-producing plasmid pT7-BLVGag (4 µg) and 5 µg each of pT7-EIAVPR, pT7-HTLV1PR or pT7-HSRVPR respectively. Cell lysates were analysed by Western immunoblotting using serum from a BLV-infected sheep. Unprocessed p46Gag and the p37Gag cleavage product are indicated on the right. The asterisk indicates predicted molecular mass of the cleavage intermediate.

Fig. 9. HeLa cells infected with recombinant vaccinia virus vTF7-3 producing bacteriophage T7 RNA polymerase were cotransfected with pT7-HIV1Gag and 1 µg pT7-HIV1PR in the absence or presence of 5 µg of pT7-HTLV1PR or pT7-HSRVPR. Cleavage was analysed by Western immunoblotting using a mouse monoclonal antibody against HIV-1 p24Gag (Hinkula et al., 1990).

dimeric PR compared to the monomer in vitro (Kräusslich, 1991). The stronger effect observed in eukaryotic cells may be due to inefficient dimerization of the monomer in vivo. These observations favour a model for PR activation predicting that PR is activated as a threshold level of PR is reached in the infected cell (Burrstein et al., 1991; Karacostas et al., 1993; Kräusslich, 1992). This may not occur until gag and pol gene products congregate at the membrane of the infected cell to bud off as virus particles.

Another reason for the inability of HIV-1 PR to efficiently cleave HIV-1 p55Gag molecules could be that we produce HIV-1 PR in the absence of Gag and Pol sequences normally present in cis on the PR-containing polyproteins. The presence of Gag and Pol sequences in cis may influence the activity of PR, and their presence may constitute a prerequisite for recognition or efficient cleavage of all sites on the Gag and Gag–Pol polyprotein. We have observed an increased activity of the HIV-1 PR when PR and the RT-part of Pol are coexpressed (Goobar-Larsson et al., 1995). The presence of RT primarily results in an increase in p24Gag levels, suggesting that RT may enhance the cleavage of 'slow' cleavage sites such as the site between p17Gag and p24Gag. In contrast, the HTLV-1 Gag precursor is efficiently processed by the HTLV-1 PR, indicating that the HTLV-1 PR may not require cis-acting sequences for efficient function. The genomic organization of HTLV-1 is such that HTLV-1 PR is produced either as a Gag–PR precursor or as a Gag–PR–Pol precursor from the viral genomic size mRNA (Kräusslich & Wimmer, 1988; Oroszlan & Luftig, 1990). It is unlikely that Pol sequences have a major effect on HTLV-1 PR activity since only a small fraction of the PR-containing molecules will be linked to...
Pol. It is also possible that the association of viral Gag proteins with genomic viral RNA may promote export and processing of Gag proteins.

We show that the N-terminal portion of HSRV Pol contain proteolytic activity, and corresponds to the HSRV PR. This has been suggested before on the basis of sequence similarity (Maurer et al., 1988). A protein encoded by this region of pol has been detected in HSRV-infected cells (Netzer et al., 1993). Although cleavage of the HSRV Gag precursor occurs in the presence of HSRV PR, the efficiency of cleavage is low. Since the identification of the PR coding region is based on similarity to other retroviral proteases, the PCR-amplified, putative PR-coding sequence used here may encode additional amino acids normally not present in mature PR. This may interfere with PR dimerization or activity. Cleavage of heterologous Gag proteins by HSRV PR cannot be detected by the assay used here. The HRSV PR shows little similarity to lentiviral PRs and PRs of the HTLV family (Aguzzi, 1993; Flügel, 1991) and may not recognize the various Gag substrates used here.

The HIV-2 PR cleaves the HIV-1 Gag precursor almost as efficiently as HIV-1 PR. Using bacterially produced HIV-1 Gag/HIV-2PR chimeras Le Grice et al. (1989) showed that HIV-1 p24Gag was more rapidly released from their HIV-1 Gag precursor using HIV-2 PR compared to using HIV-1 PR. In our system, coexpression of HIV-1 PR or HIV-2 PR with HIV-1 p55Gag gave rise to similar cleavage products, suggesting that these PRs process the same cleavage sites with similar efficiencies. This is interesting since HIV-2 PR appears to cleave out HIV-2 p28Gag/CA more efficiently than it cleaves out HIV-1 p24Gag/CA (compare Fig. 5a and Fig. 7). Therefore, HIV-2 Gag is processed more efficiently than HIV-1 Gag, suggesting that HIV-2 Gag cleavage sites are more accessible to proteolytic cleavage than HIV-1 sites. This is supported by the observation that HIV-2 Gag is subjected to proteolytic cleavage also in the absence of a retroviral PR. In this case only p43Gag was observed, indicating that not all sites could be utilized by the putative cellular protease. Surprisingly, the HIV-2 Gag was not detectably processed by the HIV-1 PR, as has been shown to occur in vitro (Töszér et al., 1991). The antiserum used here did not allow long exposures of the immunoblots and low levels of cleavage may therefore pass undetected. We may also fail to detect processing at the MA/CA junction or CA/NC junction, owing to the autocatalysis of the Gag precursor. However, translation efficiency and PR stability may vary between the various PR coding sequences and PRs. Conclusions on cleavage efficiencies must be based on the assumption that translatability and PR stability are similar among the various PRs analysed here.

Previous studies on PR cross-reactivity have compared PRs of relatively closely related viruses, and have been performed in vitro. The feline leukaemia virus PR apparently has the same specificity as murine leukaemia virus PR, and correctly processes the p65Gag precursor of Gazdar mouse sarcoma virus (Yoshinaka et al., 1985) and the Rous sarcoma virus PR was found to process the feline sarcoma virus Pr130-precursor (Khan & Stephen, 1979). Kräusslich & von der Helm reported that the avian myeloblastosis virus (AMV) PR is able to specifically digest the simian sarcoma-associated virus p76Gag precursor (Kräusslich & von der Helm, 1987). However, two intermediates arose that had no counterpart in infected cells, indicating that different sites in the Gag precursor are utilized by the different PRs. This cleavage site specificity was further demonstrated by Tomasselli et al. (1990) who showed that both the HIV-1 and avian myeloblastosis virus PR could cleave a polypeptide of the Pseudomonas exotoxin, but at different sites.

Several models for HIV-1 PR cleavage sites have been proposed (Pearl & Taylor, 1987), but none seems to faithfully predict the utilization of a site. Alignments of cleavage sites from several different retroviruses reveal an astounding heterogeneity (Oroszlan & Luftig, 1990) although observations regarding the prevalence of certain residues around the scissile bond can be made. When comparing the cleavage sequences surrounding the HTLV-1 CA protein (Oroszlan & Luftig, 1990) (N: Pro–Gln–Val–Leu*Pro–Val–Met–His; C: Thr–Lys–Val–Leu*Val–Val–Gln–Pro) with the corresponding sequences of BLV CA (N: Pro–Ala–Ile–Leu*Pro–Ile–Ser; C: Pro–Ala–Ile–Leu*Val–His–Thr–Pro), there is an apparent resemblance. The BLV Gag precursor was also efficiently processed by the HTLV-1 PR in our assay. BLV PR also show higher similarity to HTLV-1 and HTLV-2 PR than to other PRs (Nam & Hatanaka, 1986). However, the HTLV-1 PR failed to process the HIV-1 Gag precursor in spite of resemblance in the p24/p2 cleavage junction (Ala–Arg–Val–Leu*Ala–Glu–Ala–Met). This suggests that other factors besides primary amino acid sequence are involved in cleavage site utilization. Furthermore, HIV-1 and HIV-2 PR specifically cleaved the EIAV p55Gag polyprotein although there is little similarity between the proteolytic cleavage sites (Oroszlan & Luftig, 1990).

We have previously used the vaccinia virus–T7 RNA polymerase-based expression system for studies of cis-acting Gag and Pol sequences that affect activity of HIV-1 PR (Goobar-Larsson et al., 1995). The effect of various compounds on the activity of PR in eukaryotic cells may also be assessed using the vaccinia virus–T7 system. This would allow testing of PR inhibitors in human cells in the absence of infectious retrovirus. Furthermore, it should
be possible to include the T7 RNA polymerase promoter in the sense-strand PCR primer and following PCR, directly transfect the PCR fragment in the presence of pT7-HIV1Gag. Experiments to test the feasibility of this approach are in progress. Since HIV-1 variants resistant to PR inhibitors have been observed (El-Farrash et al., 1994), this may be important for the rapid assessment of the appearance of PR inhibitor-resistant viruses in patients.

In conclusion, we have shown that expression of retroviral Gag proteins and their cognate PRs in trans in eukaryotic cells results in cleavage of the Gag protein. The vaccinia virus-T7 expression system can be used as an efficient functional assay for retroviral PRs and may be used to study the properties of retroviral PRs in eukaryotic cells.


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