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Disulfide-Linked Integrase Oligomers Involving C280 Residues Are Formed In Vitro and In Vivo but Are Not Essential for Human Immunodeficiency Virus Replication

Julien Bischerour,[1] Hervé Leh,[2] Eric Deprez,[1] Jean-Claude Brochon,[1] and Jean-François Mouscadet[1]*

CNRS UMR8532, LBPA, Ecole Normale Supérieure de Cachan, 94235 Cachan,[1] and Bioalliance Pharma SA, 75015 Paris,[2] France

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The human immunodeficiency virus type 1 integrase (IN) forms an oligomer that integrates both ends of the viral DNA. The nature of the active oligomer is unclear. Recombinant IN obtained under reducing conditions is always in the form of noncovalent oligomers. However, disulfide-linked oligomers of IN were recently observed within viral particles. We show that IN produced from a baculovirus expression system can form disulfide-linked oligomers. We investigated which residues are responsible for the disulfide bridges and the relationship between the ability to form covalent dimers and IN activity. Only the mutation of residue C280 was sufficient to prevent the formation of intermolecular disulfide bridges in oligomers of recombinant IN. IN activity was studied under and versus nonreducing conditions: the formation of disulfide bridges was not required for the in vitro activities of the enzyme. Moreover, the covalent dimer does not dissociate into individual protomers on disulfide bridge reduction. Instead, IN undergoes a spontaneous multimerization process that yields a homogenous noncovalent tetramer. The C280S mutation also completely abolished the formation of disulfide bonds in the context of the viral particle. Finally, the replication of the mutant virus was investigated in replicating and arrested cells. The infectivity of the virus was not affected by the C280S IN mutation in either dividing or nondividing cells. The disulfide-linked form of the IN oligomers observed in the viral particles is thus not required for viral replication.

The insertion of a proviral DNA copy of the human immunodeficiency virus (HIV) genome into the host cell DNA is a key step of the retroviral cycle: it ensures expression and perpetuation of the viral genome (4). The process is carried out by the viral integrase (IN), encoded by the viral *pol* gene. IN catalyses two independent and successive reactions. The first reaction, 3′ processing, consists of the cleavage of both 3′ dinucleotides from each strand of the linear viral DNA, leaving overhanging CA ends. In the second reaction, strand transfer, the 3′-processed ends are used as nucleophilic agents to attack phosphodiester bonds on opposite strands of the target DNA, resulting in the joining of processed 3′ ends to 5′ phosphates in the target DNA (4). HIV IN is a 288-amino-acid protein composed of three functionally distinct domains (1). (i) The N-terminal domain contains a nonconventional HHCC zinc finger and contributes to the multimerization of the protein. (ii) The central catalytic core domain contains the D,D35E motif essential for catalysis. (iii) The C-terminal domain is involved in nonspecific binding of IN to DNA. The active form of integrase is a multimer: pairs of inactive proteins carrying mutations in different domains complement each other (13, 25). Further evidence for IN multimerization in vivo was provided by transcomplementation of IN in defective virions (16, 18). Despite extensive studies of the oligomeric status of the enzyme in solution, the precise oligomerization state required for the various steps of the integration process remains unknown. As deduced for the consensus dimer of the core domain, the distance between the two active sites is not compatible with the 5-bp gap which separates the two integration sites (15). This suggests that the minimal functional oligomer is a tetramer. The three isolated domains as well as proteins consisting of two domains are capable of forming dimers. Higher-order oligomers are observed only in solutions of the full-length protein (6, 9, 12, 19, 20, 27). Discordant findings in vitro concerning the exact nature of the oligomeric form can be explained in part by the sensitivity of oligomerization to the purification process. For instance, IN was mainly monomeric when purified in the presence of detergent whereas dimers and tetramers were recovered in the absence of detergent (10, 19). Nevertheless, recombinant IN prepared under reducing conditions has always been obtained as noncovalent oligomers. In contrast, recent reports suggested that covalent disulfide-linked oligomers of IN are present in virions (2, 23). We aimed to identify the residues responsible for the disulfide bridging in viral particles and to clarify the relationship between the formation of covalent dimers and IN activity both in vitro and in the context of the viral replication. We show that IN from a baculovirus expression system and from viral particles can form disulfide-linked oligomers. The mutation of the cysteine 280 residue is sufficient to abolish this disulfide bridging. However, the activity of the wild-type and C280S mutant are similar, and the virus infectivity in either dividing or noncycling cells was not affected by a C280S IN mutation. Thus, the disulfide-bridged form of the IN oligomers found in the viral particles is not essential for viral replication.

* Corresponding author. Present address: CNRS UMR8532, Institut Gustave Roussy, PR2, 39 rue Camille Desmoulins, 94805 Villejuif, France. Phone: 33 (0)1 42 11 50 43. Fax: 33 (0)1 42 11 52 76. E-mail: jfm@igr.fr.

MATERIALS AND METHODS

Plasmids and vectors. The *Bam*HI-*Nco*I fragment from pET-15b-IN (19) containing the coding sequence for His-tagged HIV-1 integrase (HBX2 strain) was inserted between the *Bgl*II and *Nco*I sites of the pACGP67B baculovirus transfer vector (Pharmingen). The gp67 secretion signal sequence was removed from the resulting vector, pACGP67-His-IN, by eliminating the *Spe*I-*Bam*HI fragment. The open reading frame for IN was maintained by inserting the synthetic double-strand DNA linker ADAP obtained by annealing the complementary single-stranded oligonucleotides ADAPA (5′ CTAGTAGGCGGAGC TCAGG 3′) and ADAPB (5′ GATCCCTGAGCTCCGCCTA 3′), yielding *Bam*HI and *Spe*I cohesive end. Single-amino-acid substitutions yielding C56S, C65S, C130S, and C280S mutations were introduced by site-directed mutagenesis (QuikChanger kit; Stratagene), and the presence of mutations in each case was verified by DNA sequencing. The constructs (pAC-IN and the corresponding mutants) were cotransfected with BaculoGold DNA into Sf9 cells to produce recombinant baculoviruses (BaculoGold transfection kit; Pharmingen).

The viral genomic clone containing the mutation IN(C280S) was obtained by cloning the *Eco*RI-*Nco*I DNA fragment from the pBRU2 viral genomic clone into the pGEM-T vector (Promega). The C280S substitution was constructed as described above and verified by DNA sequencing. The final vector, pBRU2-IN(C280S), was obtained by reinserting the fragment bearing the mutation into pBRU2.

Production and purification of IN. High Five cells (Invitrogen) were infected with recombinant baculoviruses and harvested 45 h later. The cells were washed in ice-cold phosphate-buffered saline and resuspended in ice-cold buffer (20 mM Tris-HCl [pH 8], plus 1 M NaCl supplemented with an antiprotease cocktail) (Roche Diagnostics, Meylan, France). They were lysed in a French press (1,000 lb/in^2) and centrifuged (40 min at 12,000 × *g* and 4°C). The supernatant was filtered (0.45-μm-pore-size filter) and incubated for 2 h with Ni-NTA–agarose beads (Qiagen). The beads were washed twice with 10 volumes of ice-cold buffer A (20 mM Tris-HCl [pH 8,] 1 M NaCl), six times with 10 volumes of buffer A containing 50 mM imidazol, and twice with 10 volumes of buffer A containing 100 mM imidazol. His-tagged IN was then eluted from the beads with buffer A supplemented with 50 μM $ZnSO_4$ and 1 M imidazol. The imidazol was removed by dialysis against a storage buffer containing 20 mM Tris-HCl (pH 8), 1 M NaCl, 50 μM $ZnSO_4$, and 10% (vol/vol) ethylene glycol. The sample was aliquoted and rapidly frozen at −80°C.

IN activity assay. IN activity assays were performed as previously describes (19). Gels were analyzed using a STORM Molecular Dynamics PhosphorImager (Pharmacia Biosciences).

Cells and viruses. All culture media were supplemented with 10% fetal calf serum and penicillin-streptomycin. HeLa and P4 (HeLa-$CD4^+$-βGal) cells were grown in Dulbecco's minimal essential medium. CEM4fx were grown in RPMI 1640 medium. CEM4fx cells were derived from the human lymphoid cell line CEM (ATCC CCL119) and express high levels of CD4 antigen. Viral stocks were obtained by transfection of HeLa cells with pBRU2 and pBRU2-IN(C280S) using Superfect (Qiagen). Virus was harvested 72 h posttransfection. For infection experiments, CEM4fx or P4 cells or peripheral blood lymphocytes (PBL) were infected with filtered cell-free virus-containing supernatants in 96-well plates at 10,000 cells/well (p24 production). Viral supernatants were removed 2 h after infection. De novo viral production was estimated by the p24 enzyme-linked immunosorbent assay (ELISA) (DuPont-NEN).

Anisotropy decay measurements. Fluorescence experiments were performed as previously described (10, 11). Excitation and emission wavelengths were 298 and 350 nm, respectively. Fluorescence experiments were performed at 25°C in 20 mM Tris-HCl buffer (pH 7.2) containing 200 nM IN, 5 mM $MgCl_2$, and 50 mM NaCl under reducing (5 mM dithiothreitol [DTT]) or nonreducing conditions. Analysis was performed by the maximum-entropy method (3).

RESULTS AND DISCUSSION

HIV-1 IN can form disulfide-linked covalent oligomers. Disulfide bridge-linked oligomers have never been described for purified HIV IN. However, bacterial expression systems that are widely used for production of recombinant IN do not allow the formation of such bridges. We investigated whether covalent oligomers may form in vitro following the production of IN in a eukaryotic insect expression system using a baculovirus vector. The HIV-1 IN gene was cloned in a modified transfection vector, pACGP67B, and the recombinant baculovirus coding for the full-length IN was used for infection of insect cells (see Materials and Methods). The recombinant protein was purified by nickel affinity chromatography under nonreducing conditions. The samples were heated in loading buffer either in the presence or in the absence of DTT, a reducing agent which cleaves disulfide bridges, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). In the presence of DTT, only one protein product, with an apparent molecular mass of 32 kDa, was revealed by silver staining (Fig. 1A, lane 1). This band corresponds to the monomer of IN as confirmed by Western blot analysis (Fig. 1B, lane 3). In contrast, in the absence of DTT (lanes 2 and 4), two major protein species were observed with apparent molecular masses of 32 and 65 kDa. They correspond to monomeric and dimeric IN, respectively. A third, minor species corresponding to a higher-order oligomer was also observed. The migration of this latter species suggested that it is a trimeric form, although it was not possible to exclude a tetramer. The yield of trimer was repeatedly either low or undetectable (compare Fig. 1 and 2). In contrast, the proportion of dimer was consistently close to half the product. This suggests a nonspecific phenomenon for trimer formation, depending on initial conditions, and in particular on the protein concentration. Mass spectrometry analysis of the purified enzyme confirmed that these multimeric forms were IN homomer (data not shown). The stable IN oligomers were reduced to monomers in the presence of DTT, demonstrating that disulfide bridging of monomers had taken place in the baculovirus expression system.

We checked that the disulfide bridges were formed inside the insect cells before the protein extraction and thus were not the consequence of artifactual disulfide bond formation after cell lysis. To monitor the amount of disulfide-bridged oligomer formed within cells, the thiol blocking agent *N*-ethylmaleimide (NEM) was added to the cell lysis buffer. Alkylation of thiols by NEM during the cell lysis ensured that disulfide bonding could not occur during the purification process (8, 21). In the absence of β-mercaptoethanol, the proportion of the IN product in the dimeric form was constant and was unchanged by the presence of different concentrations of NEM during cell lysis (Fig. 1C, lanes 4 to 6). Covalent dimers were reduced to monomers on treatment with β-mercaptoethanol (lanes 1 to 3). Recombinant IN therefore formed disulfide-linked complexes inside the insect cells prior to cell lysis. Interestingly, the alkylation treatment resulted in the accumulation of disulfide-linked trimers. Illegitimate disulfide bridges may undergo isomerization with free thiol groups when proteins are isolated in the absence of free thiol-blocking agents (8, 21). Accordingly, in the absence of thiol-blocking agents, nonspecific trimers may undergo a rearrangement to form more stable dimers. In the presence of the thiol-blocking agent, any such rearrangement would be prevented. Thus, the formation of trimers in the presence of NEM, but not in its absence, suggests that these oligomers are less specific and stable than dimers.

Cysteine 280 is responsible for the disulfide bridging in vitro. IN encoded by the genomic clone HBX2 contains six cysteine residues scattered among the three domains of the protein: two in the N-terminal domain at positions 40 and 43; three in the catalytic core domain at positions 56, 65, and 130; and one in the C-terminal domain at position 280. To identify

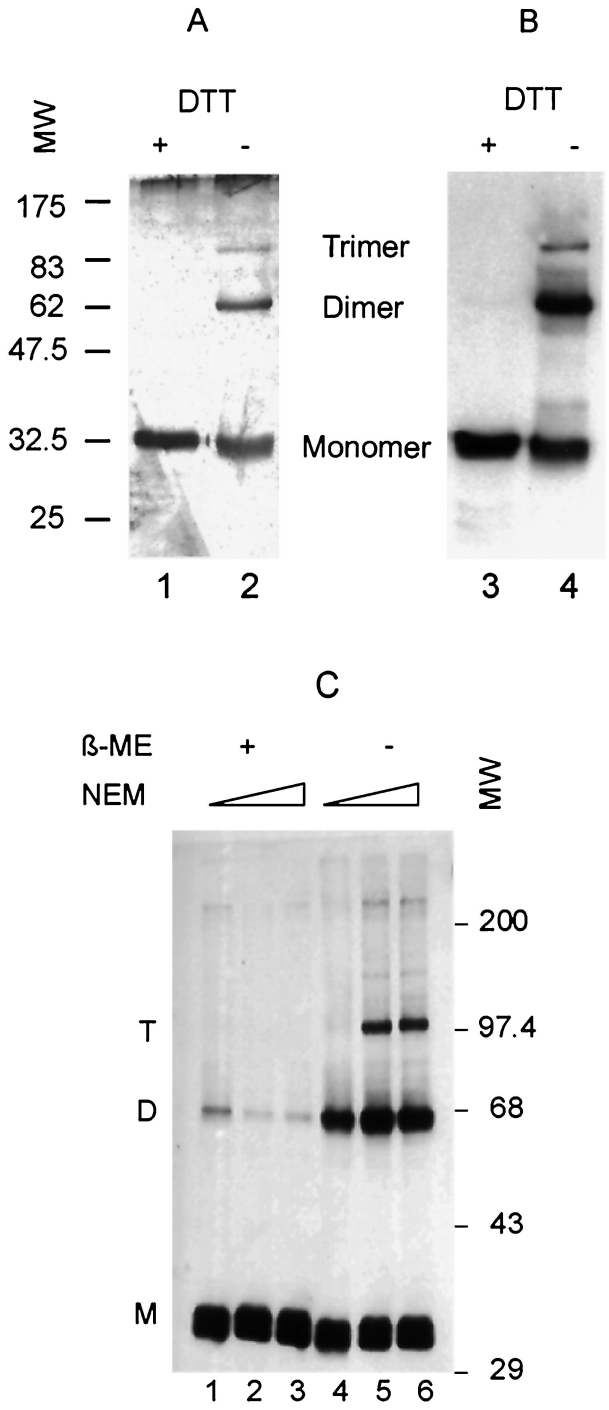


FIG. 1. IN produced in insect cells forms disulfide-linked dimers. (A and B) His-tagged IN was purified by nickel affinity in the absence of a reducing agent and subsequently analyzed by SDS-PAGE in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 10 mM DTT. (A) Proteins were revealed by silver staining. (B) Proteins were revealed by western blotting using an anti-IN polyclonal antibody. Molecular masses (MW) in kilodaltons are indicated on the left. (C) IN was purified from insect cells and analyzed by SDS-PAGE in the presence (lanes 1 to 3) or absence (lanes 4 to 6) of β-mercaptoethanol. Various concentrations of the thiol-blocking agent NEM were added before cell lysis. Lanes 1 and 4, no NEM; 2 and 5, 0.5 mM; 3 and 6, 5 mM. Molecular masses in kilodaltons are indicated on the right.

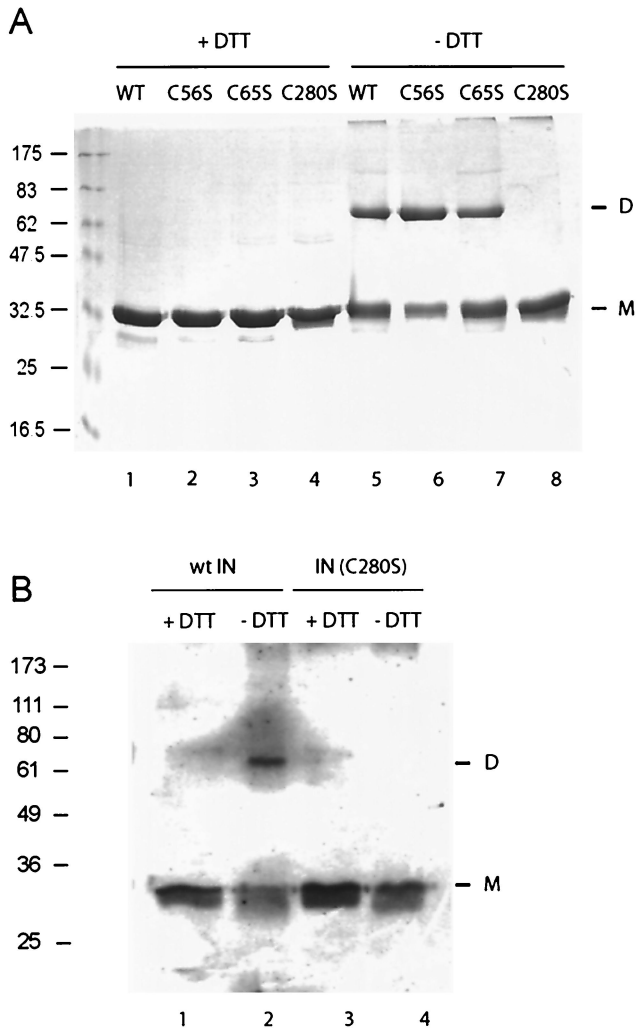


FIG. 2. Disruption of the disulfide linkage of covalent IN oligomers by mutation of cysteine 280 in vitro and in viral particles. (A) Recombinant mutant proteins were purified in the absence of reducing agents. Equivalent quantities of protein were loaded onto a 10% acrylamide gel in the presence (lanes 1 to 4) or absence (lanes 5 to 8) of 10 mM DTT. Proteins were revealed by silver staining. Molecular weights in kilodaltons are indicated on the left. M, monomer; D, dimer; WT, wild type. (B) Oligomeric status of IN(C280S) in the viral particles. Pelleted virus, corresponding to 75 ng of p24 protein, was lysed in 20 mM HEPES (pH 7.5)–150 mM NaCl–0.5% Triton X-100 buffer in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 50 mM DTT. Lysed virions were analyzed by electrophoresis on a 10% acrylamide gel and revealed by Western blotting using polyclonal antibodies against IN. Molecular masses in kilodaltons are indicated on the left. wt, wild type.

the cysteine residues engaged in disulfide bridges, four of the six cysteines were mutated into serines. Residues C40 and C43 of the N-terminal domain were not studied: there is structural and biochemical evidence that they are parts of a zinc finger-type structure (5, 6, 14). These residues coordinate a zinc ion and are therefore expected not to be available to form disulfide bridges in the native structure of IN. Indeed, substitution of cysteine 40 or 43 by alanine led to a loss of both integration activity and virion infectivity (7). Mutant proteins C56S, C65S, C130S, and C280S were produced in the baculovirus expres-

sion system and were purified in the absence of reducing agent. The oligomeric status of the mutant and wild-type proteins with or without DTT was analyzed by SDS-PAGE (Fig. 2A). The wild-type control (lanes 1 and 5) and C56S and C65S mutations (lanes 6 and 7) formed disulfide-linked dimers. In sharp contrast, the C280S mutation led to a complete absence of covalent oligomers. Thus, cysteine 280 is involved in the disulfide bond formation.

With the C130S mutant, the full-length protein was never obtained. Anti-IN polyclonal antibodies revealed the presence of two proteolysis products with apparent molecular masses of 16 and 18 kDa, although analysis of the IN protein sequence did not reveal a specific protease cleavage site generated by the cysteine-to-serine substitution (data not shown). A similar phenomenon was described for purified virions (23): a mutation at residue 130 caused an instability of the protein and a subsequent loss of the covalent oligomers. This residue is located within the catalytic pocket, and its mutation is known to abolish the enzymatic activity. Presumably, mutation of C130 destructures the catalytic core. This effect may be the cause of the instability of the protein in a eukaryotic expression system.

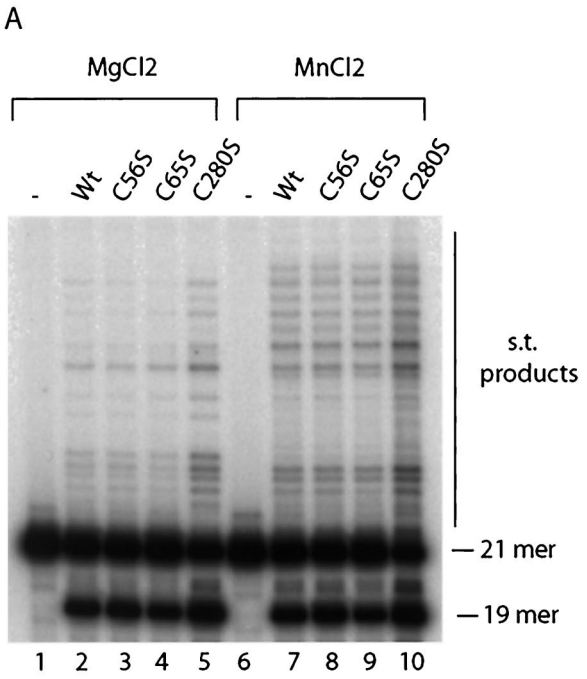
It is still not clear whether C130 is involved in covalent IN dimerization. C280 is, however, involved in the disulfide bridges, and the plausibility of a C130-C280 disulfide linkage is unclear. According to the recently solved structures of the two-domain IN proteins and the deduced model for the active tetramer, the C130 and C280 residues are too far apart to allow the formation of a disulfide bridge (9, 17, 26). In contrast, an intermolecular disulfide bridge between C280 residues is entirely plausible as models of the IN-DNA nucleoprotein lead to a significant overlap of the C-terminal domains of different protomers (17).

Cysteine 280 is responsible for the disulfide linkage formation in viral particles. IN can form covalent dimers in vitro by bridging C280 residues from independent monomers. We tested whether such disulfide-linked oligomers can be recovered from viral particles. A mutant virus bearing the IN C280S mutation was constructed, and the oligomeric status of its IN was compared to that of the wild-type enzyme. pBRU2 and pBRU2-IN(C280S), carrying BRU2 wild-type and the mutant virus, respectively, were used to transfect HeLa cells. Viral particles were recovered from the culture supernatant 72 h later. Viral production was measured as HIV-1 p24 antigen release. No quantitative differences were detected between wild-type and mutant virus, suggesting that the IN(C280S) mutation had no major impact on virus production and release (data not shown). A quantity of virus corresponding to 75 ng of p24 protein was lysed in the absence of reducing agent and analyzed by Western blotting using polyclonal anti-IN antibodies. To test for disulfide bridges, the viral extracts were heated either in the presence or in the absence of β-mercaptoethanol (Fig. 2B). In the presence of the reducing agent, comparable amounts of wild-type and mutant forms of IN were detected with an apparent molecular mass corresponding to that of the monomer. Thus, the C280S mutant IN was correctly encapsidated in the viral particles (compare lanes 1 and 3). Stable dimers of wild-type IN were observed in the absence of the reducing agent, demonstrating the presence of disulfide-bridged IN dimers in viral particles. However, no covalent dimers were observed with the C280S IN virus mutant (lanes 3 and 4) even in the absence of the reducing agent. This strongly suggests that cysteine 280 is the only residue involved in the formation of disulfide bridges in viral particles as observed in vitro.

The C280S mutation does not affect IN activity in vitro. We investigated the effects of the cysteine mutation on the catalytic activity of IN. Both 3′ processing and the subsequent strand transfer activity of the IN cysteine mutants were assayed and compared to the activity of the wild-type protein. Assays were carried out in Mn^{2+} - or Mg^{2+}-containing buffer because previous reports suggested that the oligomeric states involved in Mn^{2+}- and Mg^{2+}-dependent activities might be different (22). In the presence of a reducing agent, all the proteins were active under these conditions (Fig. 3A). However, the 3′-processing and strand transfer activity of the IN(C280S) mutant was slightly higher than that of the wild-type protein (compare lanes 2 and 5 and lanes 7 and 10). In contrast, the activities of C56S and C65S mutated proteins were not distinguishable from that of the wild-type protein. To avoid possibility that an effect of a cysteine mutation might be masked in an assay monitoring IN activity on short oligonucleotides, the experiment was repeated with the 492-bp miniviral substrate in the presence of heterologous plasmid DNA target. No further effect was observed (data not shown).

The slightly higher than wild-type activity of the C280S mutant but not the C56S and C65S mutants suggested that disulfide bridges may restrict IN activity. Thus, to determine whether the formation of disulfide linkage affects IN activity, this activity was measured in the absence or presence of concentrations of reduced glutathione, a weak thioreducing agent. In parallel to the 3′-processing assay, the oligomeric status of wild-type IN at equivalent concentrations of glutathione was monitored by Western blotting. IN(C280S) activity was not affected by the presence of glutathione, whereas the activity of wild-type IN was enhanced (Fig. 3B). This enhancement correlated with the disappearance of the disulfide-linked oligomeric forms. It thus appears that the covalent bonding between subunits in the oligomers is detrimental to the protein activity in vitro.

C280S mutation does not affect IN oligomerization. In a previous study, we showed that the ability of IN to use Mg^{2+} efficiently as a cofactor was related to its oligomerization properties in solution (19). Here we show that the disruption of the disulfide linkage increased the IN activity. We investigated the effect of the presence of a reducing agent (DTT) on oligomerization in solution by fluorescence anisotropy decay measurements. One major cause of light depolarization is the size of the fluorescent macromolecule. Accordingly, small fluorophores, characterized by fast tumbling, give rise to a fast anisotropy decay whereas large fluorophores, with slower rotational motions, yield a slower anisotropy decay. The effect of DTT addition on the IN depolarization curves is shown in Fig. 4. The anisotropy decay of the protein was significantly slower in the presence of 5 mM DTT. This indicates that the noncovalent monomeric units observed on denaturing gels have a propensity to reorganize in solution into a multimeric protein which is not cross-linked by disulfide bridges. Maximum-entropy method analysis of these decays was performed (data not shown). In the absence of DTT, IN is characterized by a broad distribution of long correlation times centered at 30 ns, sug-



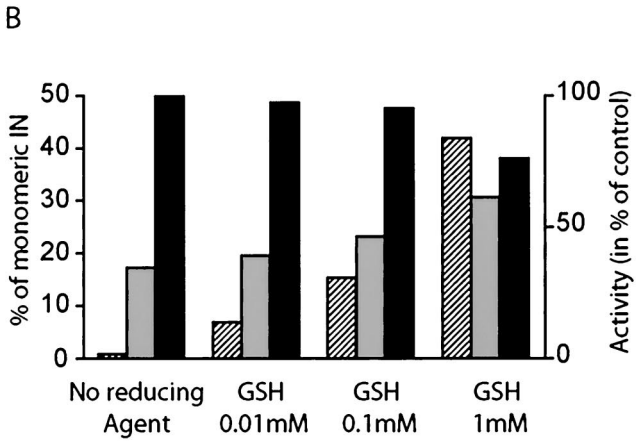


FIG. 3. Influence of the disulfide bridges on 3′-processing activity. (A) Effect of the cysteine-to-serine mutations on in vitro IN activity. Assays were performed with 3.2 nM ^{32}P-labeled U5 substrate and 100 nM purified IN incubated for 1 h at 37°C in 20 mM HEPES buffer (pH 6.8) containing 10 mM $MgCl_2$ (lanes 1 to 5) or 10 mM $MnCl_2$ (lanes 6 to 10). The reaction products were resolved on an 18% acrylamide denaturing gel. Wt, wild type. (B) Influence of disruption of the disulfide bridges on wild-type IN 3′-processing activity. In vitro assays were performed with 3.2 nM ^{32}P-labeled U5 substrate and 100 nM purified IN incubated for 1 h at 37°C in 20 mM HEPES buffer (pH 6.8) containing 10 mM $MgCl_2$ and one of a series of concentrations of reduced glutathione (GSH). The oligomeric status of wild-type IN was quantified by SDS-PAGE using densitometry analysis. The percent activity was standardized to the activity of IN(C280S) in the absence of glutathione. Striped bars indicate the percentage of monomer as assessed by denaturing gel electrophoresis; gray bars indicate the 3′-processing activity of wild-type IN; black bars indicate the 3′-processing activity of IN(C280S).

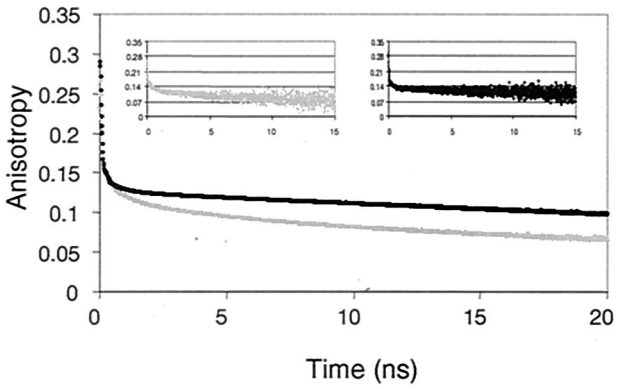


FIG. 4. Study of the oligomerization status of IN by fluorescence anisotropy decay. The fluorescence of 200 nM IN was monitored. The lifetime distribution was 0.195 ns (14%), 0.45 ns (25%), 1.3 ns (21%), 2.7 ns (27%), and 5.1 ns (13%). The figure shows the line fits resulting from maximum-entropy method analysis of the anisotropy decays (grey line, no DTT; black line, 5 mM DTT). The inset shows the corresponding experimental decays.

gesting a heterogenous solution. Addition of DTT gave rise to a well-defined long correlation time, centered at about 75 ns, consistent with a homogenous tetrameric form (10). For the C280S mutant, no significant differences were observed between reducing and nonreducing conditions (data not shown). Moreover, the decays lines obtained with the mutant and the those obtained with the wild type under reducing conditions were superimposable. These results indicate that the mutant and wild-type proteins were characterized by comparable oligomeric states in the presence of DTT. We conclude that although no functional difference was observed in vitro between the wild-type and C280S mutant IN proteins, transitory formation of covalent dimers is possible as disulfide bridges may transiently form during an early step of the enzyme production.

C280 is the residue involved in these linkages during IN production, indicating that IN monomers associate in a parallel way so that the C-terminal domains come into close proximity. In agreement, a parallel pairing is expected during the early steps of IN production because the protein is produced as part of the Gag-Pol polyprotein precursor, which is matured by the viral protease on self-activation by its parallel dimerization. This suggests that a disulfide bond may be present in a folding intermediate before being reduced in the cell to give the mature form. This type of transitory formation of disulfide linkage is not unprecedented, but its significance remains to be determined (24).

Covalent dimers are not required for HIV-1 replication either in cycling or in arrested cells. Disulfide bridges are not essential to IN activities in vitro but are present in the viral particle. We investigated whether they are required for the physiological function of IN. The replication of the virus bearing the IN(C280S) mutation was first monitored in two cellular models of dividing cells. Both CEM4fx cells and phytohemagglutinin (PHA)-stimulated PBLs were infected de novo with either the wild-type or mutant virus, obtained by transfection of HeLa. p24 production was monitored for 9 days (Fig. 5A and B). For both viruses, viral production was detectable 48 h

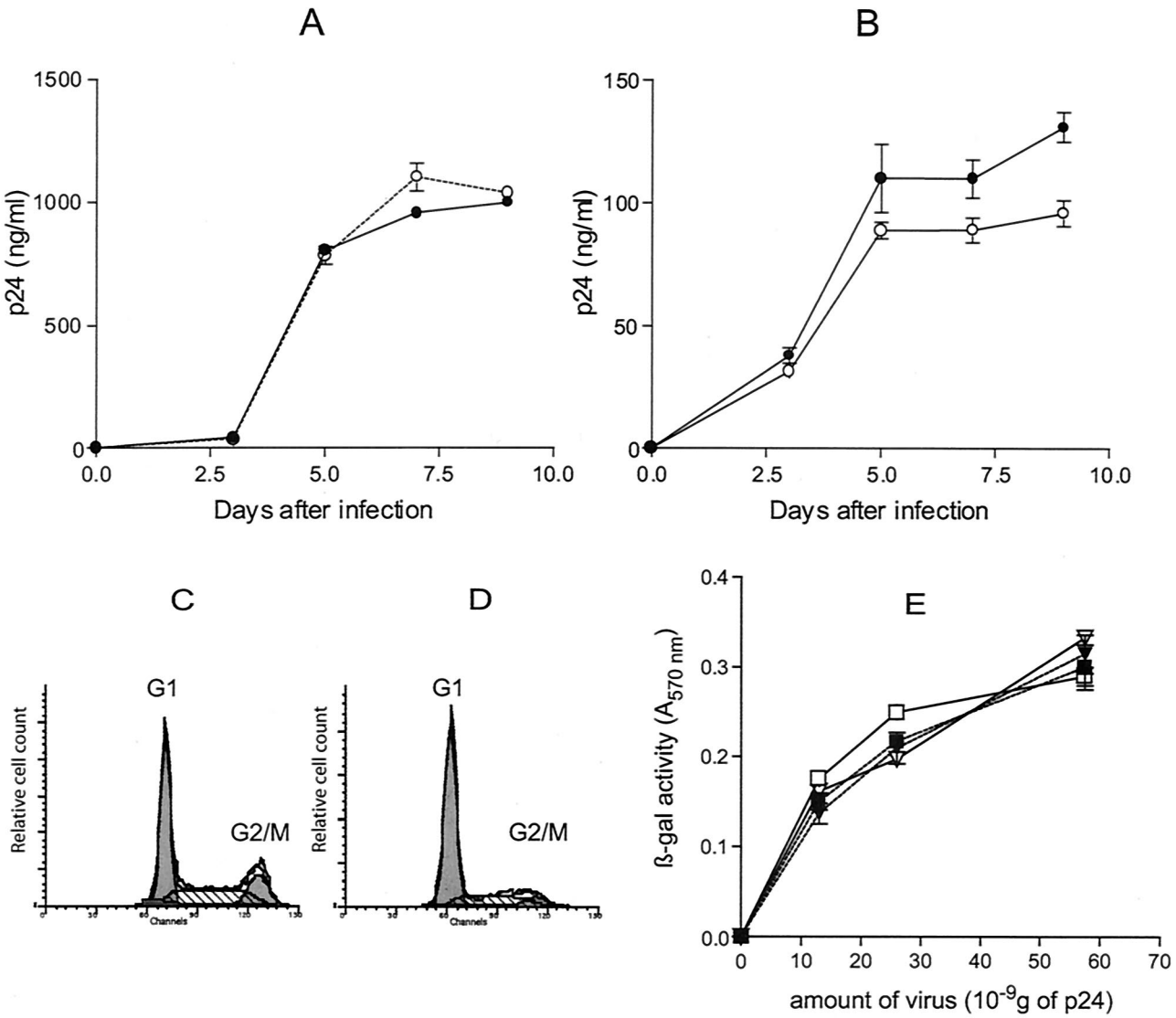


FIG. 5. Comparison of BRU2 and BRU2-IN(C280S) virus infection in dividing and nondividing cells. (A) Replication of wild-type and IN(C280S) virus in CEM4fx cells. (B) Replication of wild-type and IN(C280S) virus in PHA-stimulated PBLs. Equivalent amounts of wild-type (BRU2) (●) or mutant [BRU2-IN(C280S)] (○) viruses were used to infect CEM4fx cells (2.5 ng of p24/7,500 cells) (left) or PBLs (0.5 ng of p24/7,500 cells) (right). Virus production was monitored as the accumulation of p24 antigen in culture supernatants. p24 antigen production was assayed with the HIV p24 ELISA kit (NEN). (C and D) Cell cycle distribution of P4 cells before (C) and after (D) aphidicolin treatment (16 h). (E) Early steps of replication of wild-type and IN(C280S) virus in either P4 cells or G_1-arrested aphidicolin-treated P4 cells. The β-galactosidase (β-gal) activity of infected P4 cells was monitored after lysis and incubation with chlorophenolred-β-D-galactopyranoside (CPRG). The reaction product was quantified using a multiscan photometer at 570 nm. BRU2 (□) and BRU2-IN(C280S) (■) infection in dividing P4 cells and BRU2 (▽) and BRU2-IN(C280S) (▼) infection in nondividing aphidicolin-treated P4 cells are shown. Various quantities of virus, measured as p24 contents, were used to infect dividing or nondividing P4 cells (5×10^4 cells) for 48 h.

postinfection and peaked on day 6. The mutation had no impact on replication efficiency in CEM4Fx (Fig. 5A). Thus, the absence of disulfide linkage of IN oligomers did not impair the replication cycle in dividing cells. Viral replication was also comparable in the activated lymphocytes (Fig. 5B), although on day 5 the curve describing the mutant virus production showed a slight inflexion, giving rise to a final deviation from the wild-type curve of approximately 20%. This difference remained limited, demonstrating that destabilization of the covalent dimers did not prevent viral replication. PHA stimulation of PBLs is usually 80 to 90% effective but leaves a subpopulation of the cells in a noncycling state. Therefore, this experiment does not show that there is a true difference in the viral replication in suboptimally stimulated lymphocytes.

It has been suggested that IN dimerization may be important for the nuclear translocation of the preintegration complex (23). To address this possibility, we compared the replication of the wild-type and mutant viruses in nondividing cells. Nondividing HeLa-$CD4^+$-βgal reporter cells were blocked in the G_1 phase by aphidicolin treatment and subsequently infected with a series of different amounts of each virus. Aphidicolin was maintained throughout the infection experiment to ensure

that the cells remained arrested. Transactivation of the β-galactosidase indicator was monitored 48 h postinfection (Fig. 5C to E). Aphidicolin treatment resulted in the disappearance of G_2 cells and the accumulation of cells in the G_1 phase (compare Fig. 5C and D). β-Galactosidase activity was detected 48 h following infection with both viruses, indicating that they are able to replicate in G_1-arrested cells (Fig. 5E). Furthermore, no differences could be detected in the expression of the reporter gene regardless of whether the cells were cycling.

In conclusion, both the recombinant IN produced in insect cells and the physiological enzyme recovered from viral particles can form disulfide-linked covalent dimers. Residue C280 is the only residue responsible for the formation of the disulfide bridges. Our findings conflict with those of a previous study suggesting that covalent multimerization of IN may be important for viral replication (23). Nevertheless, we observed a small but detectable difference between the replication efficiencies of the mutant and wild-type viruses in PBLs. This may reflect a functional role in a physiological context that remains to be identified.

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