Biochemical and Biophysical Characterization of a Chimeric TRIM21-TRIM5α Protein

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The tripartite motif (TRIM) protein, TRIM5α, is an endogenous factor in primates that recognizes the capsids of certain retroviruses after virus entry into the host cell. TRIM5α promotes premature uncoating of the capsid, thus blocking virus infection. Low levels of expression and tendencies to aggregate have hindered the biochemical, biophysical, and structural characterization of TRIM proteins. Here, a chimeric TRIM5α protein (TRIM5αRh-21R) with a RING domain derived from TRIM21 was expressed in baculovirus-infected insect cells and purified. Although a fraction of the TRIM5αRh-21R protein formed large aggregates, soluble fractions of the protein formed oligomers (mainly dimers), exhibited a protease-resistant core, and contained a high percentage of helical secondary structure. Cross-linking followed by negative staining and electron microscopy suggested a globular structure. The purified TRIM5αRh-21R protein displayed E3-ligase activity in vitro and also self-ubiquitylated in the presence of ubiquitin-activating and -conjugating enzymes. The purified TRIM5αRh-21R protein specifically associated with human immunodeficiency virus type 1 capsid-like complexes; a deletion within the V1 variable region of the B30.2(SPRY) domain decreased capsid binding. Thus, the TRIM5αRh-21R restriction factor can directly recognize retroviral capsid-like complexes in the absence of other mammalian proteins.

After entering the host cell, retroviruses must evade intracellular restriction factors to establish a successful infection. The life cycle of certain retroviruses is blocked in the cells of some mammalian species prior to the initiation of reverse transcription (2, 3, 8, 42). In primates, these early blocks are mediated by TRIM5α, which binds the incoming viral capsid and promotes its premature uncoating (10, 31, 32, 38, 42, 43). Differences among the TRIM5α proteins of primate species account for the different restrictions against infection by particular retroviruses (15, 26, 35, 36, 40, 41, 43). For example, the TRIM5α proteins of Old World monkeys block human immunodeficiency virus type 1 (HIV-1), whereas the TRIM5α proteins of New World monkeys inhibit simian immunodeficiency virus (SIVmac).

TRIM5α is a tripartite motif (TRIM) family protein (28, 34). TRIM proteins contain RING, B-box 2, and coiled-coil domains. The TRIM5α protein also contains a carboxy-terminal B30.2(SPRY) domain. The contribution of the TRIM5α domain to retroviral restriction has been assessed (12, 18, 19, 30, 36, 44). The RING domain contributes to the potency of restriction but is not absolutely essential for virus inhibition (12). The RING domains of some TRIM proteins have been associated with protein ubiquitylation and targeting to the proteasome (5, 6, 16, 45). Unlike the RING domain, the B-box 2, coiled-coil, and B30.2(SPRY) domains of TRIM5α are essential for antiretroviral activity (29, 30). The coiled coil mediates TRIM5α oligomerization (13, 25), which increases the avidity of TRIM5α for the retroviral capsid (13). Capsid binding is also dependent on the B30.2(SPRY) domain (19, 37, 40). Species-specific differences in retroviral restriction often result from variation in the B30.2(SPRY) domain (18, 20, 30, 36, 44). Although the B-box 2 domain of TRIM5α is not required for capsid recognition, some changes in the B-box 2 domain result in proteins that are deficient in restricting viral infection (4, 43). It has been suggested that the B-box 2 domain contributes to an effector function involved in mediating the premature uncoating of the retroviral capsid (4).

A tendency to aggregate and low levels of TRIM5α expression present barriers to its biochemical and structural characterization. Many TRIM proteins are aggregation-prone and, when overexpressed, form pre-aggressomal cytoplasmic bodies (39). TRIM5α is expressed at low levels due to its rapid turnover (5). TRIM5α turnover is dependent on RING and B-box 2 sequences and is largely resistant to proteasome inhibitors (4). The rapid turnover of TRIM5α is not required for its antiretroviral activity (4). Substitution of the RING domain of TRIM5α with that of related TRIM proteins, which exhibit significantly longer half-lives than that of TRIM5α, results in long-lived chimeric proteins with antiretroviral function (5, 18). For example, in the present study, we examine a TRIM5α protein from rhesus monkeys (TRIM5αRh) in which the RING domain has been replaced by that of human TRIM21. This chimeric protein, designated TRIM5αRh-21R, can potently inhibit HIV-1 infection (18). Here we report on our efforts to overexpress and characterize the chimeric TRIM5αRh-21R protein.

MATERIALS AND METHODS

Plasmid construction and production of recombinant baculovirus expressing TRIM5αRh-21R variants. The recombinant baculovirus construct expressing TRIM5αRh-21R was prepared by using the pBac baculovirus expression system (Novagen). The coding sequence of TRIM5αRh-21R, also designated TRIM21-
For OSF-FL expression, the coding sequence of TRIM5Rh-21R was inserted into the pCAG vector (Invitrogen) modified to encode Streptag- and FLAG-tag epitope tags at the amino terminus of the protein, followed by a TEV protease cleavage site (16a). Compared to the OSF-FL protein, the OSF-ΔV1 protein has a deletion of the following V1 variable loop sequence: 53AEVFLLFTPSTFNFY 546 (the numbers refer to the residues of the wt-type TRIM5α protein) (24). Recombinant baculoviruses were generated by using a BaculoDirect baculovirus expression system (Invitrogen) according to the manufacturer’s protocol.

Expression and purification of TRIM5α21R variants. S9 insect cells were infected with recombinant baculovirus at a multiplicity of infection of 5 PFU/cell and incubated in serum-free SF-900 II medium for 42 h at 28°C. The cells were harvested by centrifugation at 4,000 × g for 10 min, rinsed in phosphate-buffered saline (PBS), and resuspended at 2.5 × 10^6 cells/ml in TENT buffer (100 mM Tris-HCl, 100 mM NaCl, 1% [vol/vol] Triton X-100, pH 8) in the presence of 1 mM dithiothreitol and a cocktail of protease inhibitors (at the concentrations referred to above) was added to the samples throughout each stage of the purification scheme. The final concentration of the elution peak was calculated by using ASTRA software. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) of the TRIM5α-21R protein was performed at the Yale University. Briefly, a sample containing approximately 300 μg of protein was filtered through a 0.22-μm-pore-size membrane into a 20°C-prewarmed light scattering cuvette and measurements were carried out according to the manufacturer’s instructions.

Western blotting. Aliquots were collected at each step of TRIM5α-21R purification and were resolved by SDS-PAGE. The protein was transferred to polyvinylidene difluoride Immobilon filters (Millipore) by a semidy blotting apparatus. Polycyclonal rabbit antibody directed against the purified TRIM5α-21R protein was used as the primary antibody to detect the TRIM5α-21R protein in Western blot experiments. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G was used as the secondary antibody. The Western blot was developed with an ECL chemiluminescence detection system (GE Healthcare) and the Kodak film processor system (Kodak).

Analytical size exclusion chromatography (SEC). A sample of the Q-column-purified TRIM5α-21R protein was fractionated by size exclusion on a Superdex 200 (10/30) column calibrated with molecular weight markers. The column was equilibrated in 30 mM phosphate buffer (pH 7.5)-300 mM NaCl at a flow rate of 0.4 ml/min, and the absorbance of the eluate was recorded at 280 nm. The column was mounted on a high-performance liquid chromatography system (Varian Star). Fractions were collected every minute, and a sample from each fraction was analyzed by SDS-PAGE.

SEC light scattering (SEC-LS). The molecular masses of full-length proteins were determined by Ewa Folta-Stogniew using SEC-LS in the HHMI Biopolymer Facility and W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Briefly, a sample containing approximately 300 μg of protein was filtered through a 0.22-μm-pore-size membrane into a 20°C-prewarmed light scattering cuvette and measurements were carried out according to the manufacturer’s instructions.

Sedimentation velocity. Sedimentation velocity experiments were performed in a Beckman Optima XLA analytical ultracentrifuge in an An50Ti rotor. The rotor speed was 40,000 rpm, and the temperature was maintained at 4°C. Protein-specific density and solvent density were calculated by using the software SEDNTERP (http://biochem.uthscsa.edu/aucc/software). Prior to centrifugation, the protein solution was dialyzed in PBS. The protein concentration was maintained at 0.4 mg/ml. The datasets were analyzed with SEDFIT program for a multispecies self-association and the continuous size distribution functions C(S) and C(M).

Cross-linking of the purified TRIM5α-21R protein. The purified TRIM5α-21R protein (0.4 mg/ml) was incubated with various concentrations (final concentrations of 0, 0.1, 0.3, 0.8, and 1.0 mM) of glutaraldehyde (Sigma) at room temperature for 5 min, after which excess glycerol was added to quench the reaction. The cross-linked proteins were boiled in SDS-denaturing buffer and subjected to SDS-PAGE (8% acrylamide) and Coomassie blue staining.

Electron microscopy. Purified TRIM5α-21R protein was either cross-linked with 2 mM glutaraldehyde or directly applied to carbon-coated grids after glow
purified recombinant human ubiquitin (8.5 kDa), 4 mM ATP, and 0.5 to 2.2 pmol
37°C on a rocking platform, samples were taken to test the levels of the
CA-NC proteins in a total volume of 250

and pictures were taken under low-dose conditions.

7.4], 2 mM MgCl₂, 0.1 mM dithiothreitol). After incubation (usually 60 min at
operated at 100 kV. Micrographs were recorded at a magnification of
observed in a Tecnai G2 Spirit BioTWIN (FEI Company) electron microscope
discharge. These were negatively stained with 1% (wt/vol) uranyl formate and
observed in a Tecnai G2 Spirit BioTWIN (FEI Company) electron microscope

Ubiquitylation assay. A standard assay (total volume of 30 µl) contained 0.1 µg (0.8 pmol) of purified rabbit ubiquitin-activating enzyme (8.5 kDa), 4 mM ATP; and 0.5 to 2.2 pmol of purified TRIM5Rh-21R protein in ubiquitylation buffer (20 mM Tris-HCl [pH 7.4], 2 mM MgCl₂, 0.1 mM dithiothreitol). After incubation (usually 60 min at 30°C), reactions were terminated by adding 6 µl of 6X Laemmli sample buffer. Proteins were separated by SDS-PAGE in 4 to 20% Tris-glycine precast gels (Invitrogen), transferred to polyvinylidene difluoride Immobilon-P (Millipore), and reacted with either anti-ubiquitin monoclonal antibodies (Santa Cruz) or rabbit polyclonal antibody against TRIM5Rh-21R.

CD spectroscopy. Samples for circular dichroism (CD) spectroscopy were buffer exchanged into 20 mM boric acid-NaOH (pH 8.0)–100 mM sodium fluoride by microdialysis. The CD spectrum was measured with a Jasco CD spectrophotometer (Jasco Corporation), using a quartz cuvette with a path length of 0.1 cm. Measurements were obtained in 0.5-nm intervals from 200 to 250 nm, a 1-nm bandwidth, and a 0.5-s measurement time at each wavelength. Total ellipticity was converted to mean residue molar ellipticity based on the measured concentration of samples recovered from the CD cuvette. The percentage of secondary structure was calculated by using the web-based K2D software.

CA-NC binding assay. Purification of recombinantly expressed HIV-1 capsid-nucleocapsid (CA-NC) protein from Escherichia coli was carried out as previously described (7, 19). High-molecular-weight HIV-1 capsid complexes were assembled using 300 µM CA-NC protein and 60 µM (TG)₅₀₀ DNA oligonucleotide in a volume of 100 µl of 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl. The purified TRIM5Rh-21R protein (3–7 µg) was then mixed with 5 µl of 0.3 mM CA-NC proteins in a total volume of 250 µl. After 1 h of incubation at 4, 25, or 37°C on a rocking platform, samples were taken to test the levels of the TRIM5Rh-21R and CA-NC proteins in the total input. The remainder of the reaction mixture was layered onto a 3.5-mL 70% sucrose cushion (in PBS, 5 mM dithiothreitol) and ultracentrifuged at 30,000 rpm for 1 h at 4°C in an SW55 rotor (Beckman). The sucrose cushion was then aspirated off, and the pellet was resuspended in SDS loading buffer and subsequently analyzed by SDS-PAGE. The TRIM5Rh-21R protein was detected by Western blotting with polyclonal rabbit antiserum against TRIM5Rh-21R. The HIV-1 CA-NC protein was detected by Coomassie blue staining.

RESULTS

Expression and purification of TRIM5Rh-21R. Attempts to express the wild-type human and rhesus monkey TRIM5α proteins by using a recombinant baculovirus expression vector resulted in low levels of expression in insect cells (data not shown). The chimeric TRIM5Rh-21R protein can be expressed in mammalian cells at higher levels than those attained by either TRIM5Rh or TRIM5Rhhu (18). Therefore, we expressed the TRIM5Rh-21R protein in insect cells, using a modified pBac1 vector that introduces an N-terminal His₆ sequence, S-tag, and thrombin and enterokinase cleavage sites; the estimated molecular mass of the recombinant TRIM5Rh-21R protein is 64.5 kDa (Fig. 1A). Cleared lysates from baculovirus-infected cells were added to nickel resins, and bound protein was eluted with 300 mM imidazole (Fig. 1B). The enriched protein reacted on a Western blot with an anti-TRIM5Rh-21R polyclonal antiserum (Fig. 1C). The TRIM5Rh-21R protein
was further purified on a Sepharose Q-FF anion-exchange column. The fractions containing the TRIM5Rh-21R protein were identified by SDS-PAGE and Western blotting and then pooled and analyzed by SEC. This analysis revealed the presence of higher-order aggregates (>667 kDa) and a more slowly eluting peak (<232 kDa) (Fig. 2). The latter moiety was purified by preparative SEC on a Superdex 200 HR 10/30 column (Fig. 2A and B). The identity of the TRIM5Rh-21R protein was confirmed by excision of the protein band from an SDS–polyacrylamide gel, digestion with trypsin, and MALDI-TOF analysis (data not shown). The Superdex column fractions 12 through 16 containing pure TRIM5Rh-21R protein were pooled, concentrated, and stored at 20°C until use in subsequent experiments. We found that the purified TRIM5Rh-21R protein could be stored at concentrations up to 400 g/ml at 4°C for several days without precipitating or undergoing degradation. At higher concentrations, the protein formed significant amounts of precipitate.

The TRIM5Rh-21R protein and a mutant protein containing a deletion in the V1 variable region of the B30.2(SPRY) domain were also designed with Strep and FLAG epitope tags at their amino termini. These proteins, which are designated OSF-FL and OSF-V1, respectively (Fig. 1A), were expressed in baculovirus-infected Sf21 insect cells. The OSF-FL and OSF-V1 proteins were purified using the approaches described above for the TRIM5Rh-21R protein. The proteins were at least 90% pure as determined by a using Coomassie blue-stained SDS-polyacrylamide gel (Fig. 1D).

Native state of the TRIM5Rh-21R protein. Migration of a protein on native polyacrylamide gels depends upon charge and hydrodynamic size. The pure TRIM5Rh-21R protein was mixed with nonreducing sample buffer without detergent and analyzed on a 4 to 8% Tris-glycine gradient gel. The TRIM5Rh-21R protein migrated near the 150-kDa marker, a finding consistent with the behavior of an oligomer, possibly a dimer or trimer (Fig. 3). Higher-order oligomers were also observed.

Oligomeric state of the TRIM5Rh-21R protein. The oligomeric state of the TRIM5Rh-21R protein was investigated. We utilized SEC-LS, which can estimate molecular mass independently of the Stokes radius of a protein and without reference to a calibration curve based on protein standards. In this approach, the molecular-mass determination depends only upon the light scattering and refractive indices, which are measured by detectors situated downstream of the size exclusion column. The TRIM5Rh-21R protein eluted as two peaks: the first peak contained aggregated forms of the protein, whereas the proteins in the second peak exhibited molecular masses ranging from 120 to 160 kDa (Fig. 4A and Table 1). The average molecular mass of the protein in the second peak was 139 kDa, a finding consistent with a dimer.

Additional dynamic light scattering analysis of the TRIM5Rh-21R protein indicated a diameter of approximately 57 Å and a polydispersity of 24% (Fig. 4B).

As a second approach to investigating the oligomeric state of the TRIM5Rh-21R protein, we used MS to analyze the untreated protein and the protein treated with either 2 mM glutaraldehyde or 6 M urea. In the untreated sample, monomeric, dimeric, and a trace amount of trimeric forms of the TRIM5Rh-21R protein were detected (Fig. 5A). After glutaraldehyde cross-linking, only monomeric and dimeric forms were observed (Fig. 5B); under these cross-linking conditions, no trimeric or higher-order forms (up to a molecular mass of
Thus, the purified TRIM5Rh-21R protein cross-links oligomeric forms migrated more slowly than the 150-kDa molecular mass marker. The molecular mass of the purified TRIM5Rh-21R protein was determined at different temperatures (Fig. 7A). The spectrum is characterized by a large negative change of ellipticity at 222 nm and is typical of that expected for a structured, predominantly alpha-helical, protein. A quantitative analysis of the spectra obtained at different temperatures sug-

FIG. 4. Estimation of the native molecular mass, size and polydispersity of TRIM5Rh-21R. (A) SEC-LS was used to estimate the native molecular mass of TRIM5Rh-21R. Approximately 300 µg of the purified TRIM5Rh-21R protein was applied to a Superose 6HR 10/30 column coupled with an in-line Dawn EOS laser light-scattering apparatus, refractometer, and UV detector. The solid line indicates the refractometer trace. The “dots” represent the weight-average molecular mass for each slice, measured every second. The dashed line represents the light-scattering signal at 90°. The results of the SEC-LS analysis are summarized in Table 1. (B) The polydispersity (%Pd) and size (mean and mode, in nm) of the purified TRIM5Rh-21R preparation were estimated by dynamic light scattering. A 12-µl sample of a solution of 500 µg of TRIM5Rh-21R protein/ml was analyzed by a temperature-controlled Zetasizer Nano-S dynamic light-scattering instrument at 20°C.

TABLE 1. Estimation of TRIM5Rh-21R molecular mass by SEC-LC

<table>
<thead>
<tr>
<th>Peak elution of TRIM5Rh-21R at UV tr (ml)</th>
<th>Avg mass (kDa) for the major peak</th>
<th>Range of masses (kDa) observed</th>
<th>Polydispersity (%)</th>
</tr>
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<tbody>
<tr>
<td>8.04</td>
<td>$-11 \times 10^3$</td>
<td>$&gt;3 \times 10^4$</td>
<td>ND</td>
</tr>
<tr>
<td>13.77</td>
<td>139</td>
<td>120-160</td>
<td>24.7</td>
</tr>
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* The purified TRIM5Rh-21R protein was applied to a Superose 6HR 10/30 column coupled with an in-line Dawn EOS laser light-scattering apparatus, refractometer, and UV detector as described in Materials and Methods. The molecular masses of the major peaks of eluted protein (see Fig. 4A) were estimated by using ASTRA software. ND, not determined.

The oligomeric forms of the TRIM5Rh-21R protein in the lysates of baculovirus-infected Sf9 insect cells were compared to the oligomers formed by wild-type rhesus monkey TRIM5α expressed in 293T mammalian cells, using the sulfo-EGS cross-linker. The predicted molecular mass of the TRIM5Rh-21R protein (64.5 kDa) is greater than that of wild-type TRIM5α (57.9 kDa). Despite these differences in size, cross-linking revealed that the major oligomeric state of TRIM5Rh-21R in insect cells is similar to that of wild-type TRIM5α in mammalian cells (Fig. 6B).

The TRIM5Rh-21R and OSF-FL proteins differ only in the epitope tags engineered at their amino termini (Fig. 1A). To determine whether the particular amino-terminal epitope tag influences the oligomerization of these purified proteins, the same amounts of the purified OSF-FL and TRIM5Rh-21R proteins were cross-linked with different concentrations of EGS. The cross-linked proteins were resolved on an SDS-polyacrylamide gel, which was evaluated by silver staining and Western blotting with a rabbit anti-TRIM5Rh-21R polyclonal antiserum (Fig. 6C). Both pure OSF-FL and TRIM5Rh-21R proteins cross-linked into a single species migrating slower than the 150-kDa molecular mass marker. The molecular mass difference between the two proteins, ~4 kDa, resulted in visible differences in migration of both monomeric and oligomeric species.

We conclude that the oligomeric states of TRIM5Rh-21R and TRIM5α are the same and are not apparently altered by expression in insect or mammalian cells or by the leader sequences used in the present study.

CD spectroscopy. The CD spectrum of the TRIM5Rh-21R protein was determined at different temperatures (Fig. 7A). The spectrum is characterized by a large negative change of ellipticity at 222 nm and is typical of that expected for a structured, predominantly alpha-helical, protein. A quantitative analysis of the spectra obtained at different temperatures sug-
gested that an extended alpha-helical conformation exists over a wide range of temperatures. The stability of the protein was examined by monitoring the effect of temperature on the far UV CD spectrum at 222 nm (Fig. 7B). The apparent melting temperature of the protein is 58°C.

**Topology of the TRIM5Rh-21R protein.** Transmission electron microscopy was used to examine the morphology of the purified TRIM5Rh-21R protein. Although different shapes were evident on the grid, no consistent morphology was apparent (Fig. 8A). When the TRIM5Rh-21R protein was cross-linked with glutaraldehyde at a final concentration of 2%, the protein exhibited a globular structure, with spherical particles approximately 8 to 9 nm in diameter (Fig. 8B).

**Sensitivity of TRIM5Rh-21R to proteolysis.** To investigate the domain structure and the location of solvent-accessible, flexible regions on the TRIM5Rh-21R protein, we subjected the protein to limited proteolysis by chymotrypsin and trypsin. Proteolytic intermediates were analyzed by Edman degradation or by ESI-LC/MS techniques (Fig. 9). Two prominent 39- to 42-kDa chymotrypsin-resistant fragments, designated C1 and C2, resulted from cleavages within the leader peptide and within the v1 variable region of the B30.2(SPRY) domain.
Another major protease-resistant intermediate, C3, consists of the RING, B-box 2 and coiled-coil domains, as well as the linker 2 (L2) region. A prominent chymotrypsin product, C4, includes the coiled coil, a portion of the V1 loop of the B30.2(SPRY) domain, and the intervening linker 2 (L2) region. A smaller chymotrypsin-resistant fragment, C5, corresponds to a portion of the B30.2(SPRY) domain beginning with the v1 variable loop and proceeding to the carboxyl terminus of the TRIM5<sub>Rh-21R</sub> protein.

A major trypsin-resistant fragment of 49.5 kDa, designated T3, contains the B-box 2, coiled-coil, linker 2, and the B30.2(SPRY) domains. Other major trypsin-generated fragments, T4 and T5, resulted from cleavage N-terminal to the RING and B-box 2 domains, respectively; based on the sizes of these fragments, their C termini probably occur within the linker 2 region or N-terminal part of the B30.2(SPRY) domain. Thus, the major sites available for chymotrypsin and trypsin cleavage are located near the amino-terminal end of

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**FIG. 6.** Cross-linking of the TRIM5<sub>Rh-21R</sub> protein. (A) The TRIM5<sub>Rh-21R</sub> protein, purified by anion-exchange chromatography, was incubated with the indicated concentrations (in mM) of glutaraldehyde and then boiled in Laemmli buffer and analyzed on an 8% SDS-polyacrylamide gel. M, molecular mass markers. The cross-linked band in lane 3 was cut from the gel, eluted, and analyzed by MS. The molecular mass estimation in daltons is shown above the major mass ionization peak (M = molecular mass markers). (B) Lysates from insect cells expressing the TRIM5<sub>Rh-21R</sub> protein and from 293T cells expressing wild-type rhesus monkey TRIM5α with an influenza hemagglutinin (HA) epitope tag were incubated with the Sulfo-EGS cross-linker and then boiled in Laemmli buffer and analyzed on an 8% SDS-polyacrylamide gel. A Western blot was performed using an HRP-conjugated anti-His<sub>6</sub> antibody for detection of the TRIM5<sub>Rh-21R</sub> protein and an HRP-conjugated anti-HA tag for detection of the rhesus monkey TRIM5α protein. The positions of the molecular mass markers in kilodaltons are indicated on the left. (C) Purified TRIM5<sub>Rh-21R</sub> and OSF-FL proteins were incubated with the indicated concentrations of EGS cross-linker and then boiled in Laemmli buffer and analyzed on an 8% SDS-polyacrylamide gel. The proteins were then analyzed by silver staining (left panel) and by Western blotting with an anti-TRIM5<sub>Rh-21R</sub> polyclonal antibody (right panel).
the natural TRIM5Rh-21R protein, in the interdomain linkers, and in the B30.2(SPRY) domain v1 variable region. Apparently, the TRIM5Rh-21R coiled-coil domain and the adjacent L2 linker region form a more protease-resistant core.

TRIM5Rh-21R displays E3-ubiquitin ligase activity in vitro. RING finger proteins often mediate protein ubiquitylation in the presence of certain E2 ubiquitin-conjugating enzymes (23, 24, 45). As shown in Fig. 10, the TRIM5Rh-21R protein was ubiquitylated in vitro in the presence of ubiquitin, E1, and the E2 enzyme UbcH5, as indicated by the appearance of high-molecular-mass forms that represent ubiquitin conjugates (Fig. 10, lanes 4, 5, 6, and 7). No such ubiquitylation activity was detected with bovine serum albumin (BSA) (Fig. 10, lane 2) or in the absence of either TRIM5Rh-21R protein (Fig. 10, lanes...
1 and 2) or E1 and E2 (Fig. 10, lane 3). Furthermore, a common feature of many RING finger E3 ubiquitin ligases is their ability to ubiquitylate themselves (“autoubiquitylation”). Figure 11 indicates that TRIM5Rh-21R displays autoubiquitylation in vitro. These results establish that TRIM5Rh-21R has the capacity to function as an E3 ubiquitin ligase.

**TRIM5Rh-21R binding to HIV-1 capsid complexes.** The HIV-1 CA-NC fusion protein assembles into structures that

![Diagram](http://jvi.asm.org/ Downloaded from)

FIG. 9. Sensitivity of TRIM5Rh-21R to limited proteolysis. The purified TRIM5Rh-21R protein was incubated for different time periods with the indicated proportion (weight/weight) of chymotrypsin (A) or trypsin (B). The designated fragments were analyzed either by LC/MS (ESI) or Edman degradation, as indicated. The identified peptides are listed in the boxes beneath each figure, with the numbers corresponding to the sequence position on the TRIM5Rh-21R protein of the N- or C-terminal residues. (C) Selected trypsin- or chymotrypsin-resistant peptides are aligned beneath the diagram of the complete TRIM5Rh-21R protein. The residue numbers of the TRIM5Rh-21R domain boundaries are shown above the diagram. The identified sites of proteolytic cleavage are depicted as arrows accompanied by the fragment designation.
resemble the mature viral capsid; these structures are more stable in the presence of added DNA, which serves to nucleate the CA-NC protein (7). TRIM5α proteins in cell lysates do not normally sediment through 70% sucrose gradients; however, if the TRIM5α protein specifically associates with a retroviral capsid, then the complex can pellet through a 70% sucrose cushion (43). The presence of the proteins in the input, as well as in the pellet, can then be analyzed by SDS–12% PAGE, with detection either by Coomassie blue staining (for CA-NC) or by Western blotting with a rabbit anti-TRIM5Rh-21R polyclonal serum (for TRIM5Rh-21R variants).

The OSF-FL and OSF-ΔV1 proteins were tested for the ability to bind HIV-1 CA-NC complexes at various temperatures, using different concentrations of the input TRIM5Rh-21R variant (Fig. 12A and B). The OSF-FL protein associated with the pelleted HIV-1 CA-NC complexes efficiently at 4, 25, and 37°C (Fig. 12B). When the HIV-1 CA-NC complexes were not added, the amount of the OSF-FL protein present in the pellet was reduced more than 20-fold (Fig. 12A). At all temperatures, the OSF-ΔV1 protein associated with the HIV-1 CA-NC complexes less efficiently than the OSF-FL protein (Fig. 12B). The efficiency with which the HIV-1 CA-NC complexes pelleted through the 70% sucrose cushion decreased slightly after the addition of both OSF-FL and OSF-ΔV1 proteins at 37°C (Fig. 12B and C). Subsequent studies demonstrated that this decrease in CA-NC pelleting efficiency required both the addition of a TRIM5Rh-21R variant preparation and 37°C incubation (data not shown). However, because the OSF-FL protein, which efficiently binds the HIV-1 CA-NC complexes, and the OSF-ΔV1 protein, which does not, both induce this decrease, this phenomenon appears unrelated to the biological phenotypes of these TRIM5Rh-21R variants. We conclude that TRIM5Rh-21R proteins can directly bind HIV-1 capsid-like structures, without a requirement for
other mammalian proteins. Capsid recognition is decreased by a deletion in the V1 variable region of the TRIM5 B30.2(SPRY) domain.

**DISCUSSION**

No detailed structure of any complete TRIM family member is currently available, although X-ray crystal and nuclear magnetic resonance structures of individual RING, B-box, and B30.2(SPRY) domains of some TRIM proteins have recently been determined (1, 9, 11, 21, 22, 33, 46). One roadblock to obtaining a TRIM5α structure is that, in mammalian cells, the steady-state expression levels of TRIM5α are low; in these cells, synthesis is balanced by rapid turnover, with a half-life of approximately 50 to 60 min (4, 5). This turnover is mediated mostly by pathways resistant to a variety of proteasomal inhibitors (4, 5). In the present study, we evaluated the baculovirus expression system for its utility in producing adequate quantities of recombinant TRIM5α protein for biochemical, biophysical, and structural characterization. Reminiscent of the situation in mammalian cells, expression of the wild-type human and rhesus monkey TRIM5α proteins in insect cells was also inefficient. Of interest, replacement of the TRIM5α RING domain with that of the relatively long-lived TRIM21 protein resulted in higher steady-state expression levels in both mammalian (18) and insect cells. This observation raises the possibility that the machinery involved in the rapid turnover of TRIM5α is conserved between insects and mammals.

A second roadblock to structural analysis of complete TRIM proteins is their strong tendency to form aggregates in expressing cells (34). The purified TRIM5α-21R variants studied here precipitated in insoluble aggregates at high concentrations, confirming that self-association is an intrinsic property of TRIM5α-21R and does not require stoichiometric amounts of other proteins. In addition to larger aggregates, the TRIM5α-21R protein produced in insect cells formed soluble oligomers. MS of untreated and cross-linked proteins and SEC-LS indicated that the purified TRIM5α-21R
protein is a dimer. Although estimation of the oligomeric state of the purified TRIM5Rh-21R protein by analytical ultracentrifugation was hampered by aggregation of a fraction of the protein preparation, sedimentation velocity experiments indicated that the glutaraldehyde cross-linked TRIM5Rh-21R protein exhibited a sedimentation coefficient (7.5S) and predicted molecular mass (118 kDa) consistent with those of a dimer (data not shown). Characterization of the OSF-FL protein by analytical ultracentrifugation suggests the existence of monomeric and dimeric species (16a). We conclude that these TRIM5Rh-21R derivatives form dimers.

After cross-linking with glutaraldehyde or EGS, primate TRIM5α and related TRIM proteins produced in mammalian cells migrate on SDS-polyacrylamide gels at a size predictive of trimers (13, 25, 34a). Here, we show that cross-linked forms of the purified TRIM5Rh-21R dimer migrate on SDS-polyacrylamide gels more slowly than expected. Both TRIM5Rh-21R and OSF-FL proteins exhibited similar behavior, with aberrantly slow migration of the cross-linked dimers; the monomeric forms of these proteins, in contrast, migrated on SDS-polyacrylamide gels as expected. Our experiments indicate that neither the leader peptide used for purification nor the particular cross-linker used accounts for the aberrant migration of cross-linked TRIM5Rh-21R dimers.

We also demonstrated that the cross-linked TRIM5Rh-21R protein from insect cell lysates migrated on SDS-polyacrylamide gels comparably to cross-linked TRIM5αh from lysates of mammalian cells. This result indicates that expression in insect cells did not alter the major oligomeric species formed. The simplest explanation for these observations is that TRIM5α is dimeric and that cross-linked TRIM5α dimers migrate on SDS-polyacrylamide gels in an aberrantly and misleadingly slow manner. Even the TRIM5α or TRIMCyp fragment (coiled coil and linker 2 region) minimally required for oligomerization cross-links into forms that migrate on SDS-polyacrylamide gels as apparent dimers and trimers (13); thus, the aberrantly slow migration of TRIM5α dimers appears to be a property of the structure of its oligomerization domain.

Our studies suggest that TRIM5α has a central core that is relatively resistant to protease digestion. This core contains the coiled-coil and linker 2 domains, which are known to be sufficient for TRIM5α oligomerization (13). Indeed, a core component of a natural oligomer would be expected to include the regions that surround the oligomeric axis of symmetry and contribute to multimerization. Alpha helices constitute only a minor percentage of the structures of the RING, B-box 2, and B30.2(SPRY) domains of TRIM proteins (1, 9, 11, 21, 22, 33, 46). The highly alpha-helical nature of the complete TRIM5Rh-21R protein, even at high temperatures, is consistent with a stable, central structure composed of helical coiled-coil and linker 2 elements.

Our results hint that the RING and B30.2(SPRY) domains, and possibly the B-box 2 domain as well, are flexibly appended on the amino and carboxyl termini of the central TRIM5α core. The untreated TRIM5Rh-21R oligomers exhibited no apparent uniformity by electron microscopy. In contrast, glutaraldehyde cross-linking allowed the visualization of oligomers that exhibited a more uniform, globular appearance by electron microscopy. In some TRIM family members or TRIM isoforms, the RING and/or B30.2(SPRY) domains are absent or replaced by other domains (34). This observation is consistent with a model in which different amino- or carboxy-terminal modular domains can be appended to a central oligomeric scaffold. Such a model is consistent with the proximal packing of TRIM5α domains amino- and carboxy-terminal to the central coiled coil and may explain how some alterations in the B-box 2 domain of TRIM5α might affect capsid binding in a B30.2(SPRY) domain-dependent manner (17).

Our results indicate that binding to the HIV-1 capsid does not require stoichiometric amounts of any protein other than TRIM5Rh-21R. Previous studies suggested the importance of oligomerization to the capsid-binding ability of TRIM5αh (13). Dimeric contacts between TRIM5α oligomers and the HIV-1 capsid may contribute avidity to this interaction. Dimerization of cyclophilin A has been shown to increase HIV-1 capsid binding and to create an HIV-1 restriction factor (13a, 47a). The observed reduction in binding of the OSF-ΔV1 protein to the HIV-1 CA-NC complexes, relative to that seen for the OSF-FL protein, is consistent with the results of several studies suggesting that the TRIM5α B30.2(SPRY) domain is the major determinant of the specificity and affinity of capsid recognition (18, 20, 30, 36, 44). Our results support a model in which the TRIM5α B30.2(SPRY) domains directly contact the HIV-1 capsid (25).

The TRIM5Rh-21R protein exhibited the ability to self-ubiquitylate in the presence of appropriate E1 and E2 enzymes. Ubiquitin ligase activity has been shown to be important for the function of some other TRIM proteins (14, 16, 23, 27, 45), but its role in TRIM5α-mediated restriction is uncertain. Some degree of retrovirus-restricting ability is retained by TRIM5α mutants deleted of the RING domain (12, 30), which has been demonstrated to be essential for the ubiquitin ligase activity of TRIM5 and other TRIM proteins (47). Ubiquitin addition to the capsid proteins of retroviruses restricted by TRIM5α has not been observed (31, 43). Ubiquitin ligase activity may play a role in TRIM5α turnover. The TRIM5 RING domain is important for the rapid turnover of this protein, which is in part mediated by the proteasome (4, 5). Since the half-life of TRIM5 is only modestly affected by proteasome inhibitors, however, the requirement of ubiquitylation for the major fraction of TRIM5 turnover is still uncertain.

The availability of methods for producing and purifying TRIM5 derivatives and the initial characterization of these proteins should expedite studies of their structure and mechanism of action in restricting retroviral infection.

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