The interaction between lens epithelium-derived growth factor (LGF) and human immunodeficiency virus type 1 (HIV-1) integrase (IN) is essential for HIV-1 replication. Homogeneous time-resolved fluorescence resonance energy transfer assays were developed to characterize HIV-1 integrase dimerization and the interaction between LGF and IN dimers. Using these assays in an equilibrium end point dose-response format with mathematical modeling, we determined the dissociation constants of IN dimers (Kd, 67.8 nM) and of LGF from IN dimers (Kd, 10.9 nM). When used in a kinetic format, the assays allowed the determination of the on- and off-rate constants for these same interactions. Integrase dimerization had a k_on of 0.1247 nm⁻¹·min⁻¹ and a k_off of 0.0080 min⁻¹ resulting in a K_dimer of 64.5 pm. LGF binding to IN dimers had a k_on of 0.0285 nm⁻¹·min⁻¹ and a k_off of 0.2340 min⁻¹ resulting in a K_d of 8.2 nm. These binding assays can also be used in an equilibrium end point competition format. In this format, the IN catalytic core domain produced a K_d of 15.2 nm while competing for integrase dimerization, confirming the very tight interaction of IN with itself. In the same format, LGF produced a K_d value of 35 nm when competing for LGF binding to IN dimers. In summary, this study describes a methodology combining homogeneous time-resolved fluorescence resonance energy transfer and mathematical modeling to derive the affinities between IN monomers and between LGF and IN dimers. This study revealed the significantly tighter nature of the IN-IN dimer compared with the IN-LGF interaction.

Targeting the viral integration process with small molecules as a strategy to inhibit HIV-1 replication has recently yielded an important new class of antiviral drugs. One integrase strand transfer inhibitor (INSTI), raltegravir (MK-0518), has been approved by the Food and Drug Administration for treatment of HIV-1 infection, and a second drug, elvitegravir (GS-9137), is in late stage clinical development. Based on binding experiments (1) and molecular modeling (2), strand transfer inhibitors are thought to interact with a pocket in the active site of integrase that is formed after 3'-processing of the viral DNA ends. INSTIs thus prevent the integrase-viral DNA complex from engaging host target DNA. More recently, a second site on integrase that represents the binding site for the cellular cofactor LGF was demonstrated to be a viable and attractive target for antiviral drug discovery (3–5). A small molecule inhibitor targeting this second site may retain activity against viral mutants resistant to INSTIs and complement the antiviral activity of INSTIs, akin to non-nucleoside reverse transcriptase inhibitors with nucleoside reverse transcriptase inhibitors. Hence, compounds that interact with the LGF binding pocket on IN could be used in combination with INSTIs to decrease the likelihood of resistance emergence.

The cellular cofactor LGF has been identified as the dominant binding partner of HIV-1 integrase in human cells (6–10). LGF interacts with integrase primarily through an Ñ8-amino acid domain termed the integrase binding domain (IBD) (11). A solution structure of the IBD has been derived (12). In addition, several co-crystal structures involving IBD and IN domains have been solved and include the following: IBD bound to a dimer of HIV-1 integrase catalytic core domain (CCD), IBD bound to an HIV-2 two-domain integrase (N-terminal domain (NTD) and catalytic core domain), and IBD bound to a maedi-visna virus two-domain (NTD + CCD) integrase (13–15). Several lines of evidence point to the requirement of LGF for HIV-1 replication as a viral cofactor as follows. 1) Mutations in integrase that preserve the catalytic activity of the enzyme but cause defects in viral replication also disrupt integrase interaction with LGF (16–18). 2) Suppression of LGF expression mediated by small interfering RNA alters the choice of target sites for HIV integration (19) and inhibits HIV replication (20, 21). 3) Mouse embryonic fibroblasts knocked out genetically for LGF resist infection by vesicular stomatitis virus glycoprotein G-pseudotyped HIV-1 vectors and suppress gene-specific integration (22). 4) Overexpression of the IBD in target cells inhibits HIV replication through a dominant negative mechanism (23). 5) Serial passaging of HIV-1 in cell lines overexpressing IBD selected for mutant viruses that can overcome IBD-mediated inhibition and whose integrase are mutated to bind LGF with higher affinity than IBD (24). Together, these lines of evidence validate

1 To whom correspondence should be addressed: Gilead Sciences, Inc., 333 Lakeside Dr., Foster City, CA 94404. Tel.: 650-522-5860; Fax: 650-522-5143; E-mail: MTsiang@gilead.com.
2 The abbreviations used are: HIV-1, human immunodeficiency virus; TYPE 1; LEDGF, lens epithelium-derived growth factor; IN, integrase; HTRF, homogeneous time-resolved fluorescence resonance energy transfer; CCD, catalytic core domain; INSTI, one integrase strand transfer inhibitor; IBD, integrase binding domain; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; rTEV, recombinant tobacco etch virus; MES, 4-morpholineethanesulfonic acid; DTT, dithiothreitol; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NTD, N-terminal domain; TPAsCl, tetraphenylarsonium chloride.
the disruption of the integrase-LEDGF interaction as a strategy with therapeutic potential.

Despite an abundance of structural information about the interaction of integrase with the IBD of LEDGF, precise determination of the equilibrium and kinetic binding constants between integrase monomers and between integrase and LEDGF is lacking. To characterize these interactions in detail, we developed a novel methodology that combines homogeneous time-resolved fluorescence resonance energy transfer assays and mathematical modeling. This methodology enabled the precise determination, for the first time, of the equilibrium constants and kinetic constants of integrase dimer formation and integrase-LEDGF association. These assays can also be used in an equilibrium end point competition binding format to study inhibitors of integrase dimerization or of integrase-LEDGF interaction.

**EXPERIMENTAL PROCEDURES**

**Peptides**—All the peptides used in this study were custom-synthesized and purified to >90% purity using the services of AnaSpec Inc. (San Jose, CA).

**Construction of LEDGF Expression Vectors**—Isolation of the LEDGF cDNA and the construction of pLEDGF-6His, a plasmid expressing a C-terminally His<sub>6</sub>-tagged LEDGF protein, have been described previously (25). All subsequent LEDGF variants were created using pLEDGF-6His as the starting plasmid. Plasmid pLEDGF, containing native LEDGF, was constructed by substituting a C-terminal 472-bp fragment of pLEDGF-6His with the corresponding fragment lacking the His<sub>6</sub> tag. The two PCR amplification primers used for this construction are as follows: Stu<sub>5</sub>p-LEDGF1126, 5′-CATTGAGGCCTTGGATG-3′, and Hind3p-LEDGF1593, 5′-TTTAAGCTTTAGTTATCTAGTGAAGATCC-3′. Plasmid pLEDGF-FLAG containing a C-terminally FLAG-tagged LEDGF was constructed by substituting the same 472-bp fragment of pLEDGF-6His with one containing a FLAG epitope using the two PCR amplification primers as follows: Stu<sub>5</sub>p-LEDGF1126, 5′-CATTGAGGCCTTGGATG-3′, and Hind3p-LEDGF1593, 5′-TTTAAGCTTTAGTTATCTAGTGAAGATCC-3′. Plasmid pLEDGF-FLAG was constructed by substituting the same 472-bp fragment of pLEDGF-6His with one containing a FLAG epitope using the two PCR amplification primers as follows: Stu<sub>5</sub>p-LEDGF1126, 5′-CATTGAGGCCTTGGATG-3′, and Hind3p-LEDGF1593, 5′-TTTAAGCTTTAGTTATCTAGTGAAGATCC-3′. After PCR amplification, the amplification products were purified using the QiAquick PCR purification kit (catalogue number 28104, Qiagen, Valencia, CA) and digested with Stul and HindIII. The double-digested PCR products were purified on a 1% agarose gel and cloned into the NdeI and HindIII sites of the modified pET28a vector. The digested and cloned vector was named p8His-Tev-IN-FLAG. The construct was introduced into BL21(DE3) One Shot cells (catalogue number C6000-03, Invitrogen) for protein expression.

**Construction of INCCD(F185K) Expression Vector**—The N-terminally His<sub>6</sub>-tagged integrase (6His-IN) has been described previously (2). For C-terminally FLAG-tagged IN (IN-FLAG) expression, the sequence of wild-type IN was cloned as an N-terminal His<sub>6</sub>-tagged and C-terminal FLAG-tagged protein. The N-terminal His<sub>6</sub> tag serves as an affinity purification handle that was subsequently cleaved off by rTEV protease to generate IN-FLAG. The cloning primers are as follows: NdelspIN, 5′-GCCCATATGTTTATGAGGATG-3′, and HindIII3pFLAGIN, 5′-AAGCTTTAGTTATCTAGTGAAGATCC-3′. The integrase sequence was amplified from pET3a-6HisIN as described previously (2). After amplification, the amplification products were purified using the QiAquick PCR purification kit (catalogue number 28104, Qiagen, Valencia, CA) and digested with NdeI and HindIII. The NdelspIN and HindIII3pFLAGIN inserts were purified on a 1% agarose gel and cloned into the NdeI and HindIII sites of the modified pET28a vector that encodes two more histidine residue and an rTEV recognition site (catalogue number V351-20, Invitrogen). The construct is named p8His-Tev-IN-FLAG. The construct was introduced into BL21(DE3) One Shot cells (catalogue number C6000-03, Invitrogen) for protein expression.

**Construction of INCCD(F185K) Expression Vector**—The integrase catalytic core domain (INCCD), residues 50–212 of IN, with a single solubility mutation (F185K) was cloned as an N-terminally His<sub>6</sub>-tagged protein in the plasmid p6His-INCCD(F185K). For production of untagged INCCD, the INCCD was cloned downstream of a thrombin cleavage site preceded by a His<sub>6</sub> tag in pET28a to generate the construct p6His-IIa-INCCD(F185K). The cloning primers for this construct are as follows: NdelsINCore5p, 5′-TTTCTACATTCGGATG-3′, and Hind3INCore3p, 5′-TTTAAGCTTTAGTTATCTAGTGAAGATCC-3′. After PCR amplification, the amplification products were purified using the QiAquick PCR purification kit (catalogue number 28104, Qiagen, Valencia, CA) and digested with NdeI and HindIII. The double-digested PCR products were purified on a 1% agarose gel and subcloned into pLEDGF75–6His in which the Stul and HindIII cut fragment has been removed. The construct was introduced into Escherichia coli BL21(DE3) (catalogue number C6000-03, Invitrogen) for protein expression.

**Construction of MBP-IBD Expression Vectors**—Plasmid pMAL-IBD-(347–471)-WT contains the maltose-binding protein fused to the N terminus of the wild-type LEDGF integrase binding domain IBD-(347–471). The DNA segment corresponding to IBD-(347–471) was amplified by PCR using pLEDGF-6His as template and subcloned into the Ncol- and HindIII-cloning sites of the pMAL vector (catalogue number E8200S, New England Biolabs, Beverly, MA) that encodes an rTEV protease recognition site at the C terminus of MBP. The two PCR primers are as follows: NcolIBD5p, 5′-CCATGGGGCTCAATGATTCTCGAC-3′, and Hind3-IBD3p, 5′-AAGCTTTATTTAGCTTCTCTCGTTCTTTTTG-3′. Plasmid pMAL-IBD-(347–471)-2Mut, containing MBP-IBD-(347–471) with the double mutation I365A/D366N, was made by site-directed mutagenesis of pMAL-IBD-(347–471)-WT using the QuikChange<sup>®</sup> site-directed mutagenesis kit (catalogue number 200519, Stratagene, La Jolla, CA). The sequences of the plus and minus strand mutagenic primers are as follows: IBD I365A/D366N plus, 5′-GATTAAAAATCTACAAAGCTAATATACTGTGTAGAC-3′, and IBD I365A/D366N minus, 5′-GTTCACATCAGATTATAGCTTCTTGATGGAAGAATTTTAATCC-3′.
grown overnight at 37 °C with shaking at 250 rpm. These overnight cultures were used to inoculate 6 liters of L-broth containing ampicillin (kanamycin for pHis-Tev-IN-FLAG) at an initial density of 0.05 A600/ml. The cultures were grown at 37 °C and 250 rpm until they reached a density of A600 = 0.8–1.0, which took ~3–4 h. Protein expression was then induced by addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM. The cultures were kept at 30 °C and 250 rpm for an additional 3 h before the cells were pelleted and stored at −80 °C. Sixteen grams of cell pellet were lysed in 200 ml of Lysis Buffer by passing three times through a microfluidizer. Lysis Buffer A (25 mM Tris, pH 7.0) was used for LEDGF, Lysis Buffer B (20 mM Tris, pH 7.8, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol) for MBP-IBD, Lysis Buffer C (20 mM HEPES, pH 7.0, 500 mM NaCl, 5 mM β-mercaptoethanol) for INCCD, and Lysis Buffer D (20 mM Tris, pH 8.0, 10 mM MgCl2, 2 mM β-mercaptoethanol) for 8His-Tev-IN-FLAG. The homogenate was centrifuged at 33,000 × g for 60 min at 4 °C. For most proteins, supernatant (S1) was saved for protein purification using a three-column method.

**Purification of Native LEDGF and LEDGF-FLAG**—The supernatant (S1) derived from the centrifugation of cell homogenates (see above) was loaded at 5 ml/min to an XK 26 × 20 column (catalogue number 17-0510-10, GE Healthcare) containing 20 ml of Q-Sepharose-HP that was pre-equilibrated in Buffer A (10 mM sodium phosphate, pH 7.0, 10% glycerol, 1 mM DTT) to eliminate nonspecific bacterial protein. The flow-through from the Q-Sepharose-HP column was loaded directly onto a 5-ml heparin column. Nonspecific proteins were washed away with 8–10 column volumes of Buffer A at 5 ml/min. LEDGF or LEDGF-FLAG were next eluted with a 100-ml NaCl gradient (0–1000 mM) with a slope of 10 mM/ml. Three milliliter fractions were collected during the elution. The protein peak fractions were analyzed on a NuPAGE™ 4–12% BisTris gel in MES buffer. Fractions containing the ~58-kDa LEDGF or LEDGF-FLAG band were pooled. This pool was then diluted 10 times with SP buffer. The fractions corresponding to 70–240 mM imidazole containing the LEDGF or LEDGF-FLAG band were pooled and concentrated for INCCD, and Lysis Buffer D (50 mM Tris, pH 8.0, 10 mM MgCl2, 2 mM β-mercaptoethanol) for 8His-Tev-IN-FLAG. The homogenate was centrifuged at 33,000 × g for 60 min at 4 °C. For most proteins, supernatant (S1) was saved for protein purification using a three-column method.

**Purification of MBP-IBD-(347–471)-WT and MBP-IBD-(347–471)-2Mut**—The supernatant (S1) derived from the centrifugation of cell homogenates (see above) was loaded at 3 ml/min onto a 10-ml amylose resin high flow column (catalogue number E8022S, New England Biolabs, Beverly, MA) pre-equilibrated in Buffer A (20 mM Tris, pH 7.8, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). After washing off nonspecific proteins from the column at 3 ml/min with 8–10 column volumes of Buffer A, MBP-IBD-(347–471) was eluted with Buffer B (20 mM Tris, pH 7.8, 200 mM NaCl, 1 mM EDTA, 10 mM maltose, 1 mM DTT). Three milliliter fractions were collected during elution. The protein peak fractions were analyzed on a NuPAGE™ 4–12% BisTris gel (catalogue number NP0322BOX, Invitrogen) in MES buffer. The fractions containing the ~58-kDa MBP-IBD-(347–471) band were pooled and dialyzed overnight against Buffer C (5 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT). The dialyzed sample was diluted five times with Buffer D (25 mM Tris, pH 9.0, 2 mM DTT), filtered, and loaded onto an anion exchange column, QHP-HiTrap (catalogue number 17-1154-01, GE Healthcare), pre-equilibrated in Buffer D. The column was then washed with 25 ml of Buffer D until base line was reached and eluted at 5 ml/min with a 125-ml NaCl gradient (0–1000 mM) with a slope of 8 mM/ml. Three milliliter fractions were collected during the elution. The protein peak fractions were analyzed on a NuPAGE™ 4–12% BisTris gel in MES buffer. The fractions corresponding to 100 mM NaCl containing the MBP-IBD-(347–471) band were pooled and concentrated using Centriprep-30 (catalogue number 4322, Millipore, Billerica, MA) to a final volume of ~1 ml. The concentrated sample was then loaded into a 1-ml loop and injected into a 100-ml Shodex® gel filtration column (catalogue number KW2003, Showa Denko KK, Japan) pre-equilibrated in Gel Filtration Buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 10% glycerol, 1 mM DTT). One-milliliter fractions were collected and analyzed on a NuPAGE™ 4–12% BisTris gel in MES buffer. Fractions corresponding to the first half of the elution peak containing the ~58-kDa MBP-IBD-(347–471) were pooled, determined for protein concentration using the Bradford method, quickly frozen in liquid nitrogen, and stored at −80 °C.

**Purification of 6His-INCCD (F185K) and INCCD (F185K)**—The supernatant (S1) derived from the centrifugation of cell homogenates (see above) was loaded at 5 ml/min onto a 5-ml HisTrap HP column (catalogue number 17-5248-01, GE Healthcare) pre-equilibrated in Ni Buffer A (20 mM HEPES, pH 7.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 10 mM imidazole-HCl, 10% glycerol). After washing off nonspecific proteins from the column at 5 ml/min with 8–10 column volumes of Ni Buffer A, INCCD (F185K) was eluted with a 200-ml imidazole gradient of 20–500 mM with a slope of 2.4 mM/ml. Three milliliter fractions were collected during the elution. The protein peak fractions were analyzed on a NuPAGE™ 4–12% BisTris gel (catalogue number NP0322BOX, Invitrogen) in MES buffer. Fractions corresponding to 70–240 mM imidazole containing the ~20-kDa INCCD(F185K) or 6His-IIa-INCCD(F185K) band were pooled. This pool was then diluted 10 times with SP Buffer A (20 mM HEPES, pH 6.3, 1 mM DTT) adjusted to pH 6.3.
and filtered. The diluted and filtered sample was loaded at 5 ml/min onto a 5-ml cation exchange HiTrap SP-HP column (catalogue number 17-1152-01, GE Healthcare) pre-equilibrated in SP Buffer A (20 mM HEPES, pH 6.3, 1 mM DTT). The column was then washed with SP Buffer A until base line was reached and eluted at 3 ml/min with a 125-ml NaCl gradient (0–1000 mM) with a slope of 8 mM/ml. Two and a half-milliliter fractions were collected and analyzed on a NuPAGE™ 4–12% BisTris gel in MES buffer. The fractions containing 6His-INCCD(F185K) or 6His-IIa-INCCD(F185K) band were pooled and concentrated using Centriprep-30 (catalogue number 4322, Millipore, Billerica, MA) to a final volume of 1 ml. The concentrated sample was loaded into a 1-ml loop, and injected into a 100-ml Shodex gel filtration column (catalogue number KW2003, Showa Denko KK, Japan) pre-equilibrated in GF Buffer (50 mM HEPES, pH 7.5, 400 mM NaCl, 5 mM DTT). One milliliter fractions were collected and analyzed on a NuPAGE™ 4–12% BisTris gel in MES buffer. Peak fractions were pooled, aliquoted, quick frozen in liquid nitrogen, and stored at −80 °C. To generate tag free INCCD(F185K), 6His-IIa-INCCD(F185K) was cleaved with thrombin.

Purification of 8His-Tev-IN-FLAG—For 8His-Tev-IN-FLAG, pellet (P1) was saved instead. The P1 pellet was homogenized in 80 ml of Lysis Buffer D (see under “Expression of Recombinant Proteins”) containing protease inhibitor mixture (1 pellet/50 ml of buffer). 8His-Tev-IN-FLAG in the homogenate was solubilized by addition of 26.7 ml of 5 mM NaCl (1.25 mM final) and 1.64 g of CHAPS (25 mM final). The mixture was stirred for 40 min at 4 °C and centrifuged at 38,000 × g for 35 min at 4 °C. The supernatant (S2) was diluted and mixed with an equal volume of Adjustment Buffer (62.5 mM Tris, pH 8.0, 12.5 mM MgCl2, 10 mM CHAPS, 40 mM imidazole, 20% glycerol, 2.5 mM β-mercaptoethanol). This diluted S2 fraction was then loaded at 5 ml/min onto a 5-ml HisTrap HP column (catalogue number 17-5248-02, Amersham Biosciences) pre-equilibrated in Ni Buffer A (50 mM Tris, pH 8.0, 600 mM NaCl, 10 mM MgCl2, 20 mM imidazole, 10% glycerol, 10 mM CHAPS, 2 mM β-mercaptoethanol). After washing off nonspecific proteins from the column at 5 ml/min with 8–10 column volumes of Ni Buffer A, 8His-Tev-IN-FLAG was eluted with 25 column volumes of an imidazole gradient of (20–500 mM) with a slope of 3.84 mM/ml. Three-milliliter fractions were collected during the elution. The protein peak fractions were analyzed on a NuPAGE™ 4–12% BisTris gel (catalogue number NP0322BOX, Invitrogen) in MES buffer. The fractions corresponding to 200–400 mM imidazole (60 ml) containing the ~35.9-kDa 8His-Tev-IN-FLAG were pooled and dialyzed overnight in 2 liters of Ni Buffer A without imidazole at 4 °C. After dialysis, 13 ml of this pool (~45 mg) was digested for 4 h with 2.6 mg of rTEV protease at room temperature and for an additional 2 h with an additional 1.7 mg of rTEV protease. The digested protein was loaded on a 1-ml Ni HiTrap column (catalogue number 17-5247-01, Amersham Biosciences) to remove the His6 tag and any uncleaved protein. One-third of the flow-through was concentrated using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (catalogue number UFC901024, Millipore, Billerica, MA) to a final volume of ~1 ml, then loaded into a 1-ml loop, and injected into a 100-ml Shodex® gel filtration column (catalogue number KW2003, Showa Denko KK, Japan) pre-equilibrated in GF Buffer (50 mM HEPES, pH 7.5, 400 mM NaCl, 5 mM DTT). One milliliter fractions were collected and analyzed on a NuPAGE™ 4–12% BisTris gel in MES buffer. Peak fractions were pooled, aliquoted, quick frozen in liquid nitrogen, and stored at −80 °C. To generate tag free INCCD(F185K), 6His-IIa-INCCD(F185K) was cleaved with thrombin.

Binding Affinities in the Integrase-LEDGF Complex

FIGURE 1. Diagram of IN-LEDGF and IN-IN HTRF assays. The IN-LEDGF HTRF assay measures the interaction between IN and LEDGF, whereas the IN-IN HTRF assay measures the interaction between two integrase monomers. Each binding partner is either tagged with a His6 tag or a FLAG tag. Light emissions at 665 and 620 nm are measured, and the HTRF signal is calculated as the ratio of 665:620.

HTRF-based IN-LEDGF Interaction Assay—An assay was devised to measure the interaction between HIV-1 integrase and LEDGF using a homogeneous time-resolved fluorescence energy transfer format (HTRF). In this assay, we make use of an integrase that is N-terminally tagged with hexahistidine (6His-IN) and a LEDGF that is C-terminally tagged with the FLAG epitope (LEDGF-FLAG). Anti-6His-XL665 and anti-FLAG-XL620 antibodies are used to pull down the proteins. Light emissions at 665 and 620 nm are measured, and the HTRF signal is calculated as the ratio of 665:620.
anti-FLAG-EuCryptate antibodies (catalogue numbers 61HISXLA and 61FG2KLA, Cisbio-US, Inc., Bedford, MA), which bind, respectively, to the His6 and FLAG tags, were added to allow fluorescence resonance energy transfer when LEDGF interacted with integrase (Fig. 1). To test competitors in this assay, 6His-IN, LEDGF-FLAG, and competitor were mixed in 60 μl of binding buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.1% Nonidet P-40, and 1 mg/ml bovine serum albumin) in a well of a 96-well half-area white flat bottom plate (catalogue number 3693, Corning Glass). Fifteen microliters of antibody conjugates in binding buffer were then added to start equilibrium binding. The equilibrium binding mixture contained 10 nM 6His-IN, 10 nM LEDGF-FLAG, serial 2.2-fold dilutions of the competitor, 0.45 nM anti-FLAG-EuCryptate, and 10 nM anti-6His-XL665 in binding buffer. After 6 h of incubation at 4 °C, KF was added to a final concentration of 100 mM in each well to prevent quenching of europium fluorescence before reading. The plate was read in an Envision 2102 multilabel reader (PerkinElmer Life Sciences) using 320-nm excitation filter UV2 (TRF), 590-nm emission filter 1, and 665-nm emission filter 2 with dichroic mirror D400. Raw counts (in counts/s) at 665 and 620 nm were collected, and the signal was calculated as the ratio of (cps 665:620 nm) / 10,000. The signal for the competitor dose response was analyzed by curve fitting with Equation 1 after conversion of the data to % uninhibited to determine the 50% inhibitory concentration (IC50).

\[
y = \frac{100 \times [IC50]^n}{[IC50]^n + [I]^n}
\]  
(Eq. 1)

where \( y \) = % uninhibited; \( n \) = Hill coefficient, and \([I]\) = competitor concentration. The IC50 is converted to \( K_i \) using a binding model for the IN-LEDGF interaction in the presence of a competitor described under "Binding Model for IN-LEDGF Interaction." For determin-
nation of the dissociation constant $K_d$ of the IN-LEDGF complex, the concentration of LEDGF-FLAG was fixed at 10 nM, although the concentration of 6His-IN was varied from 0 to 65 nM. Although the concentration of anti-FLAG-EuCryptate was kept at 0.45 nM (suitable for binding partners in the range of 5–1000 nM), the concentration of anti-6His-XL665 was increased to 65 nM to match the highest concentration of 6His-IN used. The signal for the binding dose response was analyzed by curve fitting with Equation 2 to determine the concentration giving half-maximal signal $EC_{50}$.

$$y = M - \frac{M \times EC_{50}^n}{EC_{50}^n + [L]^n} \quad \text{(Eq. 2)}$$

where $y = 665/620$ ratio; $n = 6$ Hill coefficient; $M =$ plateau value of the 665/620 ratio reflecting the concentration of the FLAG-tagged binding partner; and $[L] =$ concentration of the His$_6$-tagged binding partner. The $EC_{50}$ value was converted to $K_d$ using a binding model for IN-LEDGF interaction described under “Binding Model for IN-LEDGF Interaction.” A peptide containing an N-terminal His$_6$ tag connected to a C-terminal FLAG tag via an α-helical linker was generated to identify false-positive inhibitors that would disrupt antibody binding or interfere with signal generation (Fig. 1). Two such peptides were designed as follows: 6His(AQ)FLAG, HHHHHHGSAQAQAQAQAQAQGSDYKDDDDDK, and 6His(INC)-FLAG, HHHHHHGS(KELQKQITKIQN)GSDYKDDDDDK. These peptides were substituted for 6His-IN and LEDGF-FLAG in binding reactions to identify false-positives.

**HTRF-based IN-IN Interaction Assay**—An assay was devised to measure the interaction between two integrase monomers using a homogeneous time-resolved fluorescence resonance energy transfer format (Fig. 1). As in the IN-LEDGF HTRF assay, one of the binding partners is 6His-IN, but the second partner is a C-terminally FLAG tagged integrase (IN-FLAG). The same antibody pair used in the IN-LEDGF HTRF was used in this assay. After 6 h of incubation at room temperature, KF was added to a final concentration of 100 mM before reading. For determination of the integrase dimer dissociation constant, IN-FLAG was used at a fixed concentration of 3 nM, although the concentration of 6His-IN was varied from 0 to 65 nM. The binding dose response was analyzed with Equation 2 to determine the $EC_{50}$ value. The $EC_{50}$ value was converted to $K_d$ using a binding model for the IN-IN interaction described under “Binding Model for IN-IN Interaction.” It should be noted that at the integrase concentrations used a maximum of 67.5 nM, and the presence of integrase tetramer, which has a dissociation constant of ~22 μM (26), is negligible (<0.068% of total integrase). For competition binding with increasing concentrations of integrase catalytic core domain or with peptides derived from the integrase dimer interface (27), both 6His-IN and IN-FLAG were used at 10 nM. The competitor dose-response curves were analyzed with Equation 1 to determine the IC$_{50}$ value. The IC$_{50}$ value was converted to $K_i$ using a competition binding model for IN-IN interaction described under “Binding Model for IN-IN Interaction in the Presence of a Competitor.”

**Kinetic Assays for IN-LEDGF and IN-IN Associations**—For the kinetic assays of IN-LEDGF and IN-IN associations, the buffer conditions were the same as in the equilibrium end point assay, except that the two binding partners (20 nM each) were first preincubated separately with both antibody conjugates (i.e. 0.45 nM anti-FLAG-EuCryptate and 10 nM anti-6His-XL665) in 37.5 μl each for 1 h at room temperature. Kinetic measurements were started after addition of 37.5 μl of one binding partner to 37.5 μl of the other binding partner. After combination of the two binding partners, the concentrations of antibody conjugates remained the same, but the concentration of each binding partner was reduced to 10 nM. Unlike the equilibrium end point measurement, the kinetic measurement did not include KF. The time course of the 6His-IN and LEDGF-FLAG association was analyzed by direct curve fitting using Equation 3 represented by Scheme 1 where the competitor C was set to zero. The time course of the 6His-IN and IN-FLAG association was analyzed by direct curve fitting using Equation 4 represented by Scheme 2.

**Binding Model for IN-IN Interaction**—The binding model for the IN-LEDGF interaction is shown as Scheme 1, where IN is 6His-IN monomer, L is LEDGF-FLAG, and C is a competitor of LEDGF for binding to integrase.

![Diagram](image)

**Figure 2. Development of IN-LEDGF and IN-IN HTRF assays.** A, concentration dose response of binding partners in the IN-LEDGF HTRF assay. B, concentration dose response of binding partners in the IN-IN HTRF assay. C, false-positive control peptide concentration dose response. The data are shown as mean of quadruplicate runs with error bars representing the standard deviation. EuCryp, EuCryptate.
**Binding Affinities in the Integrase-LEDGF Complex**

The equilibrium concentration (i.e. after 1000 min) of LBound was solved for each value of [IN]₀. To simulate a competition binding curve of [LBound] as a function of input competitor concentration, [C], for given values of $K_{\text{dimer}}$, $K_{\text{d}}$, and $K_{\text{o}}$ the initial concentrations were set in the same manner as for the binding curve, except that [IN]₀ = 10 nM and [C]₀ = 0 – 340,000 nM.

*Binding Model for IN-IN Interaction*—By taking advantage of the HTRF format, it is possible to devise a model for the IN-IN interaction that can help determine the dissociation constant of integrase dimer. The model is shown as Scheme 2 where $A = \text{IN-FLAG}$ and $B = \text{6His-IN}$ and $K_{\text{dimer}} = K_{AA} = K_{BB} = K_{AB} = k_{-1}/k_{1}$ = dissociation constant of integrase dimer. For simplicity, it is assumed that the homodimers $A_2$ and $B_2$ and the heterodimer $AB$ have the same dissociation constant. Scheme 2 can be written as a system of five differential equations as shown in Equation 4.

\[
\begin{align*}
\frac{dA}{dt} &= 2k_{-1}A_2 + k_{+}AB - k_{1}\left(\frac{A}{4}\right)\left(\frac{A}{4}\right) - k_{1}\left(\frac{A}{4}\right)
\frac{dB}{dt} &= 2k_{-1}B_2 + k_{+}AB - k_{1}\left(\frac{B}{4}\right)\left(\frac{B}{4}\right) - k_{1}\left(\frac{B}{4}\right)
\frac{dA_2}{dt} &= k_{1}\left(\frac{A}{4}\right) - k_{-1}A_2
\frac{dB_2}{dt} &= k_{1}\left(\frac{B}{4}\right) - k_{-1}B_2
\frac{dAB}{dt} &= k_{1}\left(\frac{A}{4}\right)\left(\frac{B}{4}\right) - k_{-1}AB
\end{align*}
\] (Eq. 4)

According to this model, the only species detectable by HTRF is the integrase heterodimer $AB$. To simulate a binding curve of $AB$ as a function of input $B = \text{6His-IN}$ for a given value of $K_{\text{dimer}}$, the initial concentrations were set as follows: $[A]₀ = 3.0$ nm; $[AB]₀ = 0$ nm; $[A_2]₀ = 0$ nm; $[B_2]₀ = 0$ nm; $[B]₀ = 0$ – 2000 nm. The equilibrium concentration (i.e. after 1000 min) of $AB$ was solved for each value of $[B]₀$.

*Binding Model for IN-IN Interaction in the Presence of a Competitor That Disrupts Dimer Formation*—By using the HTRF-based IN-IN interaction assay, it is possible to measure the $K_{f}$ value of a competitor (C) capable of disrupting integrase dimer formation. There are two kinds of competitors as follows: those that are capable of self-dimerization, such as a nontagged integrase CCD (Scheme 3), and those that are incapable of self-dimerization, such as integrase dimer interfacial peptides (Scheme 4) (27). The + signs in both Schemes 3 and 4 are replaced with straight lines connecting the interacting monomers for clarity.

The mathematical models for Schemes 3 and 4 are shown as systems of differential Equations 5 and 6, respectively.
\[ \frac{dA}{dt} = 2k_{-AA} + k_{+A}(AB) + k_{-A}(AC) - k_{\frac{A}{3}} \frac{A}{3} - k_{\frac{B}{3}} \frac{B}{3} - k_{\frac{C}{3}} \frac{C}{3} \]
\[ \frac{dB}{dt} = 2k_{-BB} + k_{+B}(AB) + k_{-B}(BC) - \frac{B}{3} \frac{B}{3} - k_{\frac{A}{3}} \frac{A}{3} - k_{\frac{C}{3}} \frac{C}{3} \]
\[ \frac{dC}{dt} = 2k_{-CC} + k_{+C}(AC) + k_{-C}(BC) - \frac{C}{3} \frac{C}{3} - k_{\frac{A}{3}} \frac{A}{3} - \frac{B}{3} \frac{B}{3} \]

\[ d(AB) = \frac{A B}{3} \]
\[ d(BC) = \frac{B C}{3} \]
\[ d(AC) = \frac{A C}{3} \]

(Eq. 5)

(Eq. 6)

Again, in both Schemes 3 and 4, \( AB \) is the only species detectable by HTRF. In both Schemes 3 and 4, the integrase dimer dissociation constant, \( K_{dimer} = K_{AA} = K_{BB} = K_{CC} = k_{-A} / k_1 \), has been determined separately using Scheme 2. When using competitor \( C \) capable of self-dimerization such as a nontagged integrase CCD, it was assumed that \( K_i = K_{AC} = K_{BC} = k_{-C} / k_2 \). Simulation of a competition binding curve of \( AB \) as a function of input \( C \) was done in a way similar to that of a competitor \( C \) capable of self-dimerization using Scheme 4 and system VI.

**RESULTS**

Development of HTRF-based IN-LEDGF and IN-IN Interaction Assays—The HTRF-based IN-LEDGF and IN-IN interaction assays are depicted schematically in Fig. 1. To maximize the HTRF signal, two different pairs of antibody conjugates were tested with the 6His-IN/LEDGF-FLAG and 6His-IN-INFLAG binding partner pairs. The anti-FLAG-EuCryptate/anti-6His-XL665 antibody conjugate pair is referred to as the A1/A2 pair and the anti-FLAG-XL665/anti-6His-EuCryptate antibody conjugate pair is referred to as the B1/B2 pair. Different concentrations of mixtures of 6His-IN and LEDGF-FLAG at a 1:1 molar ratio were tested against fixed concentrations of either A1/A2 or B1/B2 (Fig. 2A). From 0 to 13 nM 6His-IN/LEDGF-FLAG, a binding partner dose response can be seen with increasing HTRF signal. At concentrations higher than 13 nM, the binding partners diluted the antibodies, and the HTRF signal gradually decreased. The A1/A2 antibody pair produced the stronger signal and was used for all subsequent IN-LEDGF HTRF assays. For the 6His-IN-IN-HTRF, a stronger dose response was also seen with the A1/A2 antibody pair (Fig. 2B). Because the A1/A2 antibody pair produced stronger signals in both IN-LEDGF and IN-IN HTRF assays, we tested this antibody pair against two false-positive control peptides (Fig. 1 and Fig. 2C). Each peptide is an \( \alpha \)-helical segment bearing an N-terminal His6 and a C-terminal FLAG tag. While the 6His(AQ)FLAG peptide contains an artificial stretch of six AQ repeats, the 6His(IN)FLAG peptide contains a known \( \alpha \)-helical segment from integrase (amino acids 211–222 of integrase). Both peptides displayed a dose response with increasing HTRF signals up to 9 nM peptide and gradual antibody dilution at higher peptide concentrations. A mixture of 6His-IN/LEDGF-FLAG (both proteins at 13 nM) was used as a positive control. Either peptide at 13 nM gave an HTRF signal that is ~7-fold higher than the 13 nM IN-IN/LEDGF mixture. In competition binding studies involving the IN-LEDGF interaction, the 6His(AQ)FLAG peptide was chosen (used at 5 nM) as a control to verify that a competitor specifically disrupts the IN-LEDGF interaction.

Affinity of Integrase Monomer-Monomer Interaction Can Affect the Apparent Dissociation Constant of LEDGF and of Its Competitors from Integrase Dimer—We first simulated the integrase-binding dose response to LEDGF according to Scheme 1 by testing the effect of different integrase dimer dissociation constants, \( K_{dimer} \) (Fig. 3A). We have used a \( K_d = 10 \) nM for this simulation because the \( K_d \) of IN-LEDGF was previously reported to be ~10 nM (28). In Fig. 3A, the curve with open circles is derived from Equation 7 describing a classical ligand-receptor system using \( K_d = 10 \) nM and \( [R]_{tot} = 10 \) nM.

\[ [RL] = \frac{K_d + [L]_{tot} + [R]_{tot} - \sqrt{(K_d + [L]_{tot} - [R]_{tot})^2 + 4K_d[R]_{tot}}}{2} \]

(Eq. 7)

that the \( K_i = K_{AC} = K_{BC} = k_{-C} / k_2 \). Simulation of a competition binding curve of \( AB \) as a function of input \( C \) was done in a way similar to that of a competitor \( C \) capable of self-dimerization using Scheme 4 and system VI.
**FIGURE 3. Simulation of LEDGF binding behavior to integrase dimer.** 

*Panel A,* simulation of integrase binding dose response. The simulation was done according to Scheme 1, where \([L]_0 = 10 \text{ nM}, [C]_0 = 0 \text{ nM},\) and \(K_d = 10 \text{ nM}\) for various concentrations of integrase and different values of the integrase dimer dissociation constant, \(K_{dimer}\). 

*Panel B,* simulation of competition dose response. The simulation was done according to Scheme 1, where \([L]_0 = 10 \text{ nM}, [IN]_0 = 10 \text{ nM},\) and \(K_d = 10 \text{ nM}\) for different values of the integrase dimer dissociation constant, \(K_{dimer}\), and various concentrations of the competitor, \(C\), with different \(K_i\) values.
where \([L]_{\text{tot}}\) is the total concentration of ligands in nanomolars; \([R]_{\text{tot}}\) is the total concentration of binding sites; \([RL]\) is the concentration of receptor-ligand complex; and \(K_d\) is the affinity of the receptor-ligand interaction. In the simulation of the IN-LEDGF interaction according to Scheme 1 and system III, the receptor is LEDGF-FLAG and the ligand is 6His-IN, which needs to dimerize before it can interact with LEDGF. Like the classical binding curve, all the curves simulated with a \(K_d = 10\) nM according to Scheme 1 eventually plateau at 10 nM (i.e. the concentration of LEDGF-FLAG) but deviate significantly from it as the integrase dimer dissociation constant \(K_{\text{dimer}}\) increases (Fig. 3A). If Equation 7 for classical binding were used to analyze these simulated IN-LEDGF binding curves, only an apparent dissociation constant \(K_{\text{d,app}}\) could be determined, but it can deviate significantly from the \(K_d\) of 10 nM used for the simulation if \(K_{\text{dimer}}\) becomes >0.25 nM (Fig. 3A). The integrase dimer dissociation constant has not been determined previously, but an upper limit of 44–168 nM was provided by the affinity of an integrase dimer interfacial peptide (27). Thus, it was important to first determine the \(K_{\text{dimer}}\) for integrase before the \(K_d\) for the IN-LEDGF interaction could be derived.

To analyze competition binding, we began by simulating competition dose responses (according to Scheme 1) for competitors with three different \(K_i\) values (0.25, 25, and 2500 nM) and using three IN dimer dissociation constants, \(K_{\text{dimer}}\) (25, 80, and 250 nM), while keeping [6His-IN], [LEDGF-FLAG], and the \(K_d\) of IN-LEDGF interaction at 10 nM (Fig. 3B). Unlike the classical competition dose-response curves, as the competitor concentration increased, the signal initially increased and reached a peak and then decreased to zero as competitor concentration was further increased. According to modeling, the shape of these curves results from two simultaneous phenomena.
as follows: 1) binding of competitor to one of the two binding sites on the unbound integrase dimer, shifting the integrase equilibrium from monomer to dimer and thereby facilitating the occupation of the second binding site by LEDGF resulting in a signal increase; and 2) with increasing competitor concentration, displacement of bound LEDGF eventually becomes predominant, resulting in a signal decrease. The magnitude of \( K_{\text{dimer}} \) for integrase and \( K_i \) for the competitor can both affect the balance between these two phenomena. When simulation was performed with increasingly higher affinities for monomer-monomer interaction (i.e. smaller and smaller \( K_{\text{dimer}} \)), the characteristic peak gradually disappears when \( K_{\text{dimer}} \) decreases below 25 nM (Fig. 3B, red curves). The reason is that with a decreasing \( K_{\text{dimer}} \), which results in more stable dimers, the monomer pool becomes so small that any further shift toward dimer by addition of a competitor becomes insignificant. When the affinity of the competitor increases (i.e. decreasing \( K_i \)), displacement of LEDGF by the competitor becomes more and more effective relative to its ability to shift the equilibrium from monomer to dimer, also resulting in a gradual decrease of the peak (Fig. 3B, compare solid line to short dashed line of the same color).

Finally, for a competitor of a given \( K_i \), the IC\(_{50}\) value of the competition dose-response curve will increase with increasing \( K_{\text{dimer}} \). For example, with \( K_i = 25 \) nM, the IC\(_{50}\) values of the competition dose-response curves are 168, 363, and 997 nM for \( K_{\text{dimer}} \) values of 25, 80, and 250 nM, respectively (Fig. 3B, long dashed lines). The reason for the IC\(_{50}\) increase with larger \( K_{\text{dimer}} \) values is that with weaker integrase monomer-monomer interactions, more competitors are initially spent in shifting monomers to dimers before they are effectively used to displace LEDGF.

**Determination of the Affinity of the Antibody Conjugates for the His\(_6\) and FLAG Tags**—Because signal generation in the assay depends not only on the association of the binding partners, IN and LEDGF, but also on the affinity of the antibody conjugates for their epitopes, we determined the affinity of all four antibody conjugates (Fig. 4). The dose responses were analyzed with the classical binding model described by Equation 8, which is a modified version of Equation 7,

\[
\text{s}[\text{RL}] = \frac{\left( K_i + [\text{L}]_{\text{tot}} + R_m}{s} - \sqrt{\left( K_i + [\text{L}]_{\text{tot}} + R_m}{s}^2 + 4 K_i R_m}{2}
\]

(Eq. 8)

where the concentration of receptor-ligand complex \([\text{RL}]\) is converted into the units of the HTRF signal (i.e. ratio of 665:620 nm) by the conversion factor, \( s \) expressed in ratio/nM, and \( R_m = s[\text{RL}]_{\text{tot}} \) is the plateau of the binding curve.

It was determined that the anti-6His-XL665 antibody conjugate \( A_2 \) has a \( K_d \) of 19 pm for the His\(_6\) tag, and the anti-FLAG-EuCrypt antibody conjugate \( A_1 \) has a \( K_d \) of 376 pm for the FLAG tag (Fig. 4A). The \( K_d \) values of antibody conjugates \( B_1 \) and \( B_2 \) were similarly determined with the anti-His\(_6\) conjugate displaying again the higher affinity (Fig. 4B). Simulation showed that with \( A_2 \) set to 65.6 nM and an affinity of 20–200 pm for the His\(_6\) tag, antibody-bound 6His-IN increases linearly with increasing input 6His-IN in the range of \( 0–65 \) nM (i.e. the dose range used in our experiments) (Fig. 4C). This means that the specific activity of labeling 6His-IN with antibody does not change within the dose range used, and therefore the \( K_d \) determination of the binding partners will not be affected. The affinity of the anti-FLAG-EuCrypt is somewhat weaker, but this does not pose a concern because LEDGF-FLAG and IN-FLAG are always used at a fixed concentration.

**Subnanomolar Affinity of the Integrase Monomer-Monomer Interaction**—To determine the dissociation constant \( K_{\text{dimer}} \) of integrase dimer, 3 nM IN-FLAG was combined with varying concentrations of 6His-IN, and the HTRF signal was measured after equilibrium was reached (see under “Experimental Procedures”). The HTRF signal generated by the formation of \( AB = 6\text{His-IN-FLAG} \) heterodimer (see Scheme 2) is plotted against the concentration of 6His-IN (Fig. 5A). Equation 2 was used to fit the data to determine an EC\(_{50}\) of 5.3 nM for this interaction. This EC\(_{50}\) value is an upper estimate of the \( K_{\text{dimer}} \) because as the concentration of 6His-IN increased, most of the input 6His-IN is spent in the formation of homodimers that are not detectable by HTRF. Only a smaller fraction of total 6His-IN associates or undergoes subunit exchange with IN-FLAG at equilibrium to form the signal-generating heterodimer. To calculate the \( K_{\text{dimer}} \) in the absence of an analytical solution for the binding curve, we resorted to a numerical method by simulating a family of binding curves according to Scheme 2 and Equation 4 using \( [\text{IN-L}^{\text{tot}}] = [\text{IN-FLAG}_{\text{tot}}] = 3 \) nM and different values of \( K_{\text{dimer}} \) (0.0025, 0.025, 0.25, 2.5, and 250 nm). Each curve was then analyzed phenomenologically with Equation 2 to determine the corresponding EC\(_{50}\) (Fig. 5B). The EC\(_{50}\) values thus determined can be correlated with the \( K_{\text{dimer}} \) values using parabolic interpolation. Once the successive quadratic functions fitting three points at a time were determined by curve fitting (Fig. 5C, solid line), the quadratic function describing the curve segment bracketing the experimental EC\(_{50}\) value of 5.3 nM was used to convert this value into a \( K_{\text{dimer}} \) of 67.8 pm. Using this value of \( K_{\text{dimer}} \) we calculated that in an equilibrium solution of 10 nm integrase (monomer concentration), ~89% of integrase is in dimeric form.
Nanomolar Affinity of LEDGF Interaction with Integrase Dimer—
Having determined the integrase dimer dissociation constant, $K_{\text{dimer}}$, the $K_d$ value of LEDGF from integrase dimer was determined experimentally by fixing [LEDGF-FLAG] at 10 nM while varying [6His-IN] (Fig. 6A). Having established that the integrase monomer-monomer interaction is very tight and that the great majority of integrase is present in dimeric form, we first analyzed our binding data with Equation 7 for classical binding. Fitting Equation 7 to the data yielded an apparent $K_d$ of 11.6 nM for the interaction of LEDGF with integrase dimers. To determine the real $K_d$, we first fit Equation 2 to the data to determine an $EC_{50}$ of 19.2 nM. Next, we used parabolic interpolation to establish a correlation between $K_d$ and $EC_{50}$ by simulating a family of binding curves corresponding to different $K_d$ values according to Scheme 1 and Equation 3 while fixing $K_{\text{dimer}}$ at the value of 67.8 pM, which was determined above (Fig. 6B). Using the result of parabolic interpolation (Fig. 6C), the $EC_{50}$ of 18.8 nM was converted to a real $K_d$ of 10.9 nM. Because the integrase monomer-monomer interaction is very tight, the apparent $K_d$ of 11.6 nM determined from the classical binding equation is very close to the real $K_d$ determined according to Scheme 1.

Kinetics of IN-LEDGF and IN-IN Associations—By monitoring the kinetics of 6His-IN and LEDGF-FLAG association (Fig. 7A) in our HTRF assay format, we were able to determine the on-rate ($k_{\text{on}} = k_1$) and off-rate constants ($k_{\text{off}} = k_2 = k_3$) for LEDGF-FLAG binding to 6His-IN dimer (see Scheme 1) by assuming we have the same integrase dimer dissociation constant of 67.8 pM, which was determined previously. The $k_{\text{on}}$ for LEDGF binding to IN dimer was measured at 0.0285 nM$^{-1}$min$^{-1}$ and the $k_{\text{off}}$ at 0.234 min$^{-1}$. These on- and off-rate constants are in ranges comparable with those observed with anti-carbohydrate antibodies determined by surface plasmon resonance spec-
**Binding Affinities in the Integrase-LEDGF Complex**

The dissociation constant of LEDGF from integrase dimer calculated from the ratio of $k_{off}/k_{on}$ is 8.2 nm, which is comparable with the $K_d$ value we determined above using equilibrium end point dose-response analysis (Fig. 6A). From the off-rate constant of LEDGF dissociating from the IN dimer, we calculated a half-life for the IN-LEDGF complex of ~3 min.

Similarly, by monitoring the kinetics of 6His-IN and IN-FLAG association (Fig. 7B), we determined the on- and off-rate constants, $k_+$ and $k_-$, for integrase monomer-monomer association (see Scheme 2). The on-rate constant $k_+$ is 0.1247 nm$^{-1}$min$^{-1}$, which is ~4.4-fold larger than that of LEDGF binding to integrase dimer. The off-rate constant $k_-$ is 0.0080 min$^{-1}$, which is ~29-fold smaller than that of LEDGF binding to integrase dimer. The ratio of $k_{off}/k_{on}$, which yields an integrase dimer dissociation constant of 64.5 pm, is comparable with the $K_{dimer}$ we previously determined using equilibrium end point dose-response analysis (Fig. 5A).

From the off-rate constant of integrase monomers, we calculated a half-life for integrase dimer of ~87 min.

**Peptides and Small Molecules Are Capable of Displacing LEDGF from Integrase**—We tested in our HTRF-based IN-LEDGF interaction assay various competitors that included a tag-free native LEDGF, an MBP-IBD fusion, and a mutated version of MBP-IBD, where Ile-365 and Asp-366 previously identified as hot spot contact residues with integrase have been replaced with AN. This mutant peptide termed LEDGF(363CA-368AN) displayed a 9.4-fold decrease in competition potency (Table 1). The LEDGF-(354–378 IN)-peptide, which contains mutation D366N, was less effective by ~9.4-fold at competing with LEDGF compared with the wild-type peptide LEDGF-(354–378). Peptide LEDGF-(354–378-AD) which contains the D365A mutation displayed an ~14.5-fold lower potency compared with wild-type peptide. The inhibitory potency of peptide LEDGF-(354–378-AN), which contains the double mutation D366N/I365A, was comparable with either mutation alone indicating that the individual mutations maximally compromise the interaction of the peptide with integrase. A six amino acid circular peptide, LEDGF(363CG-368C), containing the critical integrase binding dipeptide ID motif, retained significant potency with an $IC_{50}$ of 26 nM, whereas the ID or VD dipeptides by themselves were essentially inactive with $IC_{50}$ values of ~2 nm. Three linear peptides derived from LEDGF, together with circularized versions of these peptides, were tested in the IN-LEDGF interaction assay. The circular versions seemed to be more potent in this assay compared with the linear forms (Table 1). In particular, LEDGF(363SG-368S) was not inhibitory, suggesting that circularization of the hexapeptide facilitated binding by possibly pre-organizing the ID dipeptide-binding motif into a preferred conformation. To determine whether the potency of the circular peptide LEDGF(363CG-368C) was dependent on the presence of the key ID residues required for binding to integrase, a peptide in which ID was replaced with AN was tested. This mutant peptide termed LEDGF(363CG-368AN) displayed a ~40-fold decrease in competition potency (Table 1).

Finally, to assess whether the greater flexibility provided by the glycine residue in LEDGF(363CG-368C) was responsible for the higher binding affinity of this peptide or whether the charge and longer side chain of the naturally occurring lysine residue in LEDGF(363CK-368C) was the cause of decreased potency, glycine was substituted with alanine yielding peptide LEDGF(363CA-368C). LEDGF(363CA-368C) displayed comparable affinity to LEDGF(363CK-368C), suggesting that the flexibility provided by the glycine may be responsible for the greater affinity. Another possibility that we cannot reject at this time is that the increased flexibility of the LEDGF(363CG-368C) peptide may allow it to either bind differently within the same pocket as LEDGF(363CA-368C) or to a different site entirely resulting in increased ability to disrupt the IN-LEDGF interaction.

**Figure 5. Determination of integrase dimer dissociation constant.** A, 6His-IN dose response of 6His-IN-IN-FLAG heterodimer formation. The data represent the means of two independent determinations each done in quadruplicate. Standard deviations are shown as error bars. IN-FLAG was used at 3 nm. The concentration $EC_{50}$ of 6His-IN giving half-maximal signal was determined by curve fitting using Equation 2. B, simulation of 6His-IN dose response for 6His-IN-IN-FLAG heterodimer formation. The simulation was done according to Scheme 2, where $[A] = 3$ nm for different values of the integrase dimer dissociation constant, $K_{dimer}$. The $EC_{50}$ was calculated for each simulated curve. C, correlation of $EC_{50}$ to $K_{dimer}$. According to the correlation curve, an $EC_{50}$ of 5.7 nm determined from A translates to a $K_{dimer}$ of 67.8 pm. EuCrypt, EuCryptate.
INCCD and Peptides Derived from the IN Dimer Interface Disrupt Integrase Dimers—Two peptides, INH1 and INH5, derived from the α-1 and α-5 helices located at the integrase dimer interface were previously shown to perturb the integrase oligomer equilibrium in favor of monomer species and to inhibit integrase catalytic function (27). We sought to test these two peptides in our IN dimerization HTRF assay to determine whether they can prevent the formation of the 6His-IN-IN-FLAG heterodimer. In addition, we used INSC3G, a scrambled version of INH5, into which three glycine residues were introduced to break the α-helicity as a negative control. As a positive control, we used the integrase catalytic core domain, INCCD (F185K) containing the solubility mutation F185K. INCCD(F185K) inhibited the formation of 6His-IN-IN-FLAG heterodimers with an IC₅₀ of 2.5 μM (Fig. 9A). Using Equation 5 represented by Scheme 3 and a method similar to that described in Fig. 6, B and C, this IC₅₀ value was converted to a Kᵢ value of 15.2 nM. A possible reason why the IC₅₀ of INCCD is ~164-fold larger than the Kᵢ value is that INCCD not only competes against integrase heterodimer formation, which generates the signal, but it also competes against integrase homodimer formation, which cannot be directly detected, and finally it forms self-dimers that are inactive as competitors. INH1 and INH5 but not INSC3G inhibited the formation of 6His-IN-IN-FLAG heterodimers with IC₅₀ values of 448 and 88 μM, respectively (Fig. 9B). Using Equation 6 represented by Scheme 4 and a method similar to that described in Fig. 6, B and C, the IC₅₀ values were converted to Kᵢ values of 8.9 and 1.8 μM for INH1 and INH5, respectively. For these interfacial peptides, the fold-difference between IC₅₀ and Kᵢ is smaller at ~50-fold likely because the peptides do not form self-dimers as INCCD does. The Kᵢ values of the peptides can be directly compared with the Kᵢ value of INCCD and
showed that INH5 displayed an ~118-fold lower affinity for integrase monomer than INCCD.

**DISCUSSION**

In this study, we describe HTRF-based assays coupled with mathematical models to study the interaction of integrase with itself and with LEDGF. Unlike the AlphaScreen™ method previously used to investigate the IN-LEDGF interaction (4), the HTRF method is suitable for both equilibrium end point and real time kinetic analyses. Because our system involves at least four different interactions (i.e. anti-6His-6His, anti-FLAG-FLAG, 6His-IN-6His-IN, and 6His-IN-LEDGF-FLAG), we systematically determined the dissociation constants of these four interactions.

We started with the determination of the affinity of the antibody conjugates for their respective epitopes (i.e. 6His tag or FLAG tag) because these affinities dictate whether corrections to the $K_d$ values would be necessary for the IN-IN and IN-LEDGF interactions. We showed that because of the high affinity of the anti-His6 antibody conjugate for 6His-IN (20–60 pM), no corrections to the $K_d$ values determined for IN-LEDGF or IN-IN were necessary.

We next studied the interaction of integrase monomers to form an integrase dimer. According to the co-crystal structure of INCCD bound to the IBD of LEDGF, the IN dimer corresponds to the minimal oligomeric form of integrase that serves as a binding partner for LEDGF (13). The dissociation constant of an integrase tetramer into two integrase dimers has been determined previously to be $22 \mu M$ using sedimentation equilibrium (26), but the dissociation constant of integrase dimer has never been reported to our knowledge. The closest upper estimate
for an integrase dimer dissociation constant was provided by the IC50 values (44–168 nM) of peptides derived from the integrase CCD dimer interface capable of dissociating integrase dimers (27). This information provided an estimate of the integrase concentration range to initiate our experiments and within which integrase tetramer formation is negligible. Using His-tagged and FLAG-tagged integrases and an equilibrium end point method, we determined the equilibrium dissociation constant for integrase dimer, $K_{dimer}$, of 67.8 pM. This is the first ever demonstration of a picomolar interaction between two binding partners that represent identical monomers. Other biophysical methods are effective at determining the affinity of an interacting pair when each binding partner can be produced separately and later brought together for the purpose of affinity measurements. However, when the pair is composed of subunits of a very tight homodimer that is already formed, novel methods were needed to determine the affinity of such homodimers. We made use of the sensitivity of HTRF detection, the ability of the tight IN homodimers to exchange their subunits, and mathematical modeling of the monomer-monomer association and dimer subunit exchange to accurately determine the affinity between two integrase monomers. By monitoring and analyzing the time course of integrase heterodimer species formation, we arrived at a very similar $K_{dimer}$ value of 64.5 pm with an integrase dimer half-life of ~87 min. This integrase monomer-monomer interaction is very tight in comparison with other known dimer dissociation constants as follows: 730 nM for MIP-1β (32), 4 nM for human TATA-binding protein (33), and 3.6 nM for HIV-1 protease (34). It is, however, comparable with the overall dissociation constant of 400 pm for active HIV-1 reverse transcriptase heterodimer (35, 36). Examination of the integrase CCD dimer structure revealed large shared surface areas consistent with nanomolar affinities (Fig. 10). In addition, ion pairs formed by four buried salt bridges may be giving increased affinity and stability resulting in a subnanomolar dimer dissociation constant for integrase dimers. The method described here for the determination of the $K_{dimer}$ for integrase can be potentially applied to other proteins forming high affinity dimers when it is impractical to measure by other biophysical means. Because the integrase dimer forms a very tight complex, its disruption would be very difficult and thus may not constitute a practical therapeutic strategy as demonstrated by our attempt to use INCCD and peptides derived from the integrase dimer interface. Even the INCCD showed a $K_i$ that...
is 224-fold larger than the $K_{d\text{dim}}$ while the most potent peptide derived from the integrase dimer interface showed a $K_d$ 27,000-fold larger than the $K_{d\text{dim}}$.

According to the co-crystal structure of integrase CCD and IBD, the interaction between integrase and LEDGF involves an integrase dimer with two symmetrical binding pockets formed at the dimer interface that can accommodate two molecules of LEDGF (13). This binding interaction cannot be a priori analyzed with the classical binding model of one receptor and one ligand without potentially grossly overestimating the $K_d$. With the knowledge of the integrase dimer dissociation constant, we used a binding model involving an obligate dimer with two symmetrical binding sites to analyze the equilibrium binding dose response of integrase to LEDGF. By this method, we determined that the $K_d$ value of the LEDGF interaction with integrase is 10.9 nM. Because the integrase dimer is a very tight complex, the binding of LEDGF to integrase practically behaved according to the classical one receptor, one ligand binding model. This explains why the apparent $K_d$ of 11.6 nM is comparable with the real $K_d$. By monitoring and analyzing the time course of the IN-LEDGF association, we determined a very similar $K_d$ value of 8.2 nM with a IN-LEDGF complex half-life of ~3 min. A previous deletion study revealed that in addition to the primary LEDGF binding determinant present in the integrase CCD dimer interface, the NTD of integrase also contributes to the binding affinity (7). More recent studies showed that LEDGF and IBD can promote detectable integrase tetramer formation at integrase concentrations of 1–14 μM (30, 37), and it was proposed that the integrase tetramer has two low affinity and two high affinity binding sites for the IBD of LEDGF (37). According to molecular modeling, although the two low affinity binding sites are present in the integrase dimer, the two high affinity sites involving additional NTD contacts can only be realized in the context of the integrase tetramer (37). Under the much lower integrase concentrations used in our study, it is unlikely that there is significant formation of integrase tetramers, even in the presence of LEDGF. In support of this, the $K_d$ value for the 6His-INCD (lacking the additional NTD contacts) and LEDGF-FLAG interaction was similar to that of 6His-IN interacting with LEDGF-FLAG (data not shown), suggesting that the $K_d$ determined in this study is that of the low affinity binding site.

Using the HTRF IN-LEDGF interaction assay in competition binding mode, we determined the $IC_{50}$ and $K_d$ values of variants of LEDGF and LEDGF-derived peptides. As expected, the $K_d$ value of native LEDGF determined in competition binding is comparable with the $K_d$ value of the 6His-IN and LEDGF-FLAG interaction as determined by direct binding. Consistent with previous results using the pulldown assay (12), the double mutant I365A/D366N in the binding loop of IBD decreased its affinity for integrase by ~18-fold relative to wild type in our HTRF assay. Recently, three peptides derived from the LEDGF IBD were shown to inhibit integrase activity by shifting integrase oligomer equilibrium toward tetramers (30). The $K_d$ values (0.8–4.6 μM) for two of these peptides (i.e. LEDGF (354–378) and LEDGF (360–370)) as determined in our competition binding assay correspond to the same range as the $K_d$ values previously determined by direct binding using fluorescence anisotropy (30). More recently, a shorter version of peptide LEDGF (354–378), termed LEDGF (355–377), was shown to disrupt the IN-LEDGF interaction with an $IC_{50}$ of 25 μM in

| Competitor      | Sequence | $IC_{50}$ (nM) | $K_d$ (nM) |
|-----------------|----------|---------------|------------|
| Native LEDGF    |          | 91            | 35         |
| MBP-IBD-WT      |          | 565           | 218        |
| MBP-IBD 2Mut    |          | 10,200        | 3,942      |
| LEDGF-(354–378)| WIHAIEKSLKDIINDVRCIEALD | 2,020 | 779 |
| LEDGF-(354–378 IN)| WIHAIEKSLKDIINDVRCIEALD | 19,050 | 7,376 |
| LEDGF-(354–378 AD)| WIHAIEKSLKDIINDVRCIEALD | 29,200 | 11,328 |
| LEDGF-(354–378 AN)| WIHAIEKSLKDIINDVRCIEALD | 16,900 | 6,541 |
| LEDGF-(354–378 SC) | WNSDVEDIAKCIIRDLINANLHIE | 56,833 | 22,166 |
| LEDGF-(360–370) | WNSLKDINLDV | 11,900 | 4,601 |
| LEDGF-(362G-368G) | CLKIDNLDVNC | 396,000 | 164,508 |
| LEDGF-(362G-368G) | CLKIDNLDVNC | 430,200 | 179,818 |
| LEDGF-(362G-368G) | CLKIDNLDVNC | 26,000 | 10,080 |
| LEDGF-(362G-368G) | CLKIDNLDVNC | 395,667 | 164,360 |
| LEDGF-(362G-368G) | CLKIDNLDVNC | 1,000,000 | 460,668 |
| LEDGF-(362G-368G) | CLKIDNLDVNC | 1,570,000 | 790,281 |
| LEDGF-(363C-368C) | CGIDNC | 2,596,000 | 1,506,242 |
| LEDGF-(363C-368C) | CGIDNC | 1,800,000 | 937,065 |
| LEDGF-(363C-368C) | CGIDNC | 2,199,000 | 1,210,503 |
| LEDGF-(363C-368C) | CGIDNC | 2,671,000 | 1,564,763 |
| LEDGF-(363C-368C) | CGIDNC | 514,000 | 218,072 |
| LEDGF-(363C-368C) | CGIDNC | 4,086,000 | 2,826,796 |
| LEDGF-(363C-368C) | CGIDNC | 4,338,000 | 3,083,020 |
| LEDGF-(363C-368C) | CGIDNC | 17,073,000 | 28,419,981 |

$IC_{50}$ values (0.8–4.6 μM) for two of these peptides (i.e. LEDGF (354–378) and LEDGF (360–370)) as determined in our competition binding assay correspond to the same range as the $K_d$ values previously determined by direct binding using fluorescence anisotropy (30). More recently, a shorter version of peptide LEDGF (354–378), termed LEDGF (355–377), was shown to disrupt the IN-LEDGF interaction with an $IC_{50}$ of 25 μM in
an AlphaScreen format (3). In this study, the peptide was preincubated with integrase before the addition of LEDGF in contrast to our equilibrium competition binding assay where the peptide was added to a premix of integrase and LEDGF. The 9.4- and 14.4-fold decreases in competition potency resulting from the introduction of the D366N and I365A mutations, respectively, into the LEDGF-(354–378)-peptide is consistent with the >85% inhibition of pulldown or strand transfer activity stimulation by the same mutation previously introduced into either IBD (12, 18) or full-length LEDGF (12, 25, 38).

In an attempt to identify the smallest LEDGF peptide still capable of binding integrase, we discovered that the ID and VD residues of LEDGF critical for binding integrase are devoid of binding affinity for the enzyme ($K_I$ values $>1$ mM). A dipeptide can potentially exist in several conformational states, and it is possible that the more stable conformation has the wrong shape for binding to integrase. Alternatively, additional contact sites that may be present in longer peptides could be necessary for binding. A longer linear hexapeptide containing this ID dipeptide motif, termed LEDGF-(363SG-368S), was also inactive. However, when this hexapeptide was circularized through terminal cysteine S–S bonding, the binding affinity was increased by ~70-fold suggesting that circularization of the hexapeptide may have facilitated binding by constraining the dipeptide-binding motif into a preferred binding conformation. We showed that the ID dipeptide in this context was still essential for binding as its substitution with the AN dipeptide abolished binding.

We also tested in our IN-LEDGF interaction assay, the small molecule TPAsCl, which had been shown through crystallography to interact with integrase at a site on the dimer interface, now known as the LEDGF binding pocket (31). TPAsCl proved to be a very weak competitor of LEDGF binding to integrase, displaying an IC$_{50}$ of ~400 nM, and it did not appear to interfere with antibody binding. Although this binding is very weak, it nonetheless appears specific because three small molecule HIV antiviral drugs (zidovudine, raltegravir, and tenofovir), which served as negative controls, produced IC$_{50}$ values at least 10-fold higher than that of TPAsCl in the IN-LEDGF interaction assay. This result also suggests that the assay may be amenable to screening small molecule competitors of LEDGF.

In conclusion, we have developed HTRF-based IN-IN and IN-LEDGF interaction assays and combined them with...
mathematical modeling to characterize the four major equilibrium binding constants involved in this assay system. This study is the first to determine the very tight picomolar interaction between the subunits of the integrase homodimer and the first to derive the affinity of LEDGF for integrase by using a model of ligand binding to an obligate dimer. The novel study is the first to determine the very tight picomolar inter- 

FIGURE 10. Binding Affinities in the Integrase-LEDGF Complex

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