FEBS Letters 546 (2003) 300–306 FEBS 27384

Mutations at the CXCR4 interaction sites for AMD3100 influence anti-CXCR4 antibody binding and HIV-1 entry

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Received 11 March 2003; revised 14 May 2003; accepted 21 May 2003

First published online 4 June 2003

Edited by Hans-Dieter Klenk

Abstract The interaction of the CXCR4 antagonist AMD3100 with its target is greatly influenced by specific aspartate residues in the receptor protein, including Asp^{171} and Asp^{262}. We have now found that aspartate-to-asparagine substitutions at these positions differentially affect the binding of four different anti-CXCR4 monoclonal antibodies as well as the infectivity of diverse human immunodeficiency virus type 1 (HIV-1) strains and clinical isolates. Mutation of Asp^{262} strongly decreased the co-receptor efficiency of CXCR4 for wild-type but not for AMD3100-resistant HIV-1 NL4.3. Thus, resistance of HIV-1 NL4.3 to AMD3100 is associated with a decreased dependence of the viral gp120 on Asp^{262} of CXCR4, pointing to a different mode of interaction of wild-type versus AMD3100-resistant virus with CXCR4.

Key words: CXCR4; Antibody recognition; Human immunodeficiency virus coreceptor; Bicyclam; Resistance

1. Introduction

The initial binding of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, gp120, to the cellular receptor CD4 induces a conformational change in the gp120 complex, allowing it to interact with either of the chemokine receptors CCR5 or CXCR4 [1–7]. The so-called R5 viruses, isolated from patients early in the course of HIV-1 infection, infect macrophages and T lymphocytes by using CCR5 as the coreceptor [1]. On the other hand, CXCR4 is the major co-receptor for the more pathogenic X4 HIV-1 strains that are typically T cell-tropic and very often emerge during progression from the asymptomatic stage to immunodeficiency (AIDS) [8–10].

The bicyclam AMD3100 is the most potent and specific CXCR4 antagonist described to date [11–13]. The compound strongly inhibits the replication of a wide variety of X4 HIV strains and clinical isolates in vitro [11,12], and clearly showed activity against X4 viruses in a phase II clinical trial in HIV-1-infected individuals [14]. High-affinity binding of AMD3100 to CXCR4 is based upon electrostatic interactions of the positively charged cyclam rings with Asp^{171} and Asp^{262}, located in transmembrane domain (TM) IV and TM VI, respectively, at each end of the main ligand binding crevice of CXCR4 (Fig. 1) [15]. Mutation of these negatively charged aspartic acid residues to neutral asparagines strongly impairs the CXCR4 antagonism and antiviral activity of AMD3100 [16]. However, a few other negatively charged amino acid residues, including the aspartates at positions 182 and 193 (Fig. 1), have been identified as alternative or additional interaction sites for AMD3100 ([17] and our unpublished data).

Given the unique receptor specificity and potent anti-HIV activity of AMD3100, the amino acid residues that account for the high-affinity interaction between AMD3100 and CXCR4 could also be important determinants in the interaction of HIV-1 gp120 with CXCR4. Indeed, in analogy with the net positive charge of AMD3100 and the natural CXCR4 ligand, stromal cell-derived factor 1 (SDF-1), the V3 loop of X4 HIV gp120, is also enriched in basic amino acids [18]. We have now investigated the impact of aspartate-to-asparagine substitutions at positions 171, 182, 193 and 262 of CXCR4 on anti-CXCR4 monoclonal antibody (mAb) binding and on coreceptor activity of CXCR4 for diverse X4 or R5/X4 HIV-1 strains (i.e. NL4.3, IIIB, NDK, HE and the clinical isolate CI-10), and for mutant viruses resistant to AMD3100, SDF-1 or dextran sulfate.

2. Materials and methods

2.1. Viruses

The T-tropic (X4) HIV-1 molecular clone NL4.3 was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program (Bethesda, MD, USA). The X4 HIV-1 IIIB strain was a kind gift from Dr. L. Montagnier (Pasteur Institute, Paris, France). The X4 HIV-1 strain NDK [19] was obtained from the Centralised Facility for AIDS Reagents (UK Medical Research Council). The dual-tropic (R5/X4) HIV-1 HE strain was initially isolated from a patient at the University Hospital in Leuven, and had been routinely cultured in MT-4 cells [20]. Virus stocks of the dual-tropic R5/X4 clinical isolate CI-10 were generated by coculture of peripheral blood mononuclear cells from a healthy donor with lymphocytes from an HIV-infected person. Coreceptor usage of the viruses was determined by viral replication assays in CXCR4- and CCR5-transfected U87.CD4 cells.

NL4.3 strains resistant to AMD3100 [21], SDF-1 [22] and dextran sulfate [23] were selected as described previously, by passaging the virus in MT-4 cells in the presence of progressively increasing concentrations of the antiviral agent.

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00609-4

2.2. Cell cultures

Human astroglioma U87 cells expressing human CD4 (U87.CD4) were kindly provided by Dr. Dan R. Littman (Skirball Institute of Biomolecular Medicine, New York, NY, USA) and were transfected with the different CXCR4 mutants as described previously [16]. The stable transfectants were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK) containing 10% fetal bovine serum (FBS) (BioWhittaker Europe, Verviers, Belgium), 0.01 M HEPES buffer (Invitrogen), 0.2 mg/ml geneticin (G-418 sulfate) (Invitrogen) and 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). The cell cultures were maintained at 37°C in a humidified, CO_2-controlled atmosphere and subcultivations were done every 2–3 days by digestion of the monolayers with trypsin/EDTA (Invitrogen).

2.3. Antibody stainings and flow cytometry

The antibodies used in this study were: PE-conjugated mouse anti-human CD4 mAb clone SK3 (BD Biosciences, San Jose, CA, USA), PE-conjugated rat anti-CXCR4 mAb clone 2B11 (BD Pharmingen, San Diego, CA, USA), PE-conjugated mouse anti-human CXCR4 mAb clone 12G5 (BD Pharmingen), PE-conjugated mouse anti-human CXCR4 mAb clone 44717.111 (R&D Systems Europe, Abingdon, UK), unconjugated mouse anti-human CXCR4 mAbs clones 44708.111 and 44716.111 (R&D Systems Europe) and PE-conjugated goat anti-mouse IgG Ab (Caltag Laboratories, San Francisco, CA, USA).

After trypsin digestion, CXCR4-transfected U87.CD4 cells were incubated for at least 1 h at room temperature to allow re-expression of receptor proteins at the cell surface. Then, 0.5×10^6 cells were washed once with phosphate-buffered saline (PBS) containing 2% FBS, resuspended in 100 μl PBS containing 2% FBS and incubated with the appropriate antibody for 30 min on ice. Thereafter, the cells were washed and, in case of an unlabelled primary antibody, incubated for 30 min on ice with the secondary antibody (diluted 1/100 in PBS with 2% FBS). After final washing with PBS, the cell samples were fixed in 1% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (BD Biosciences Europe, Erembodegem, Belgium). As a negative control for non-specific background staining, the cells were stained in parallel with Simultest Control γ_1/γ_{2a} (in case of directly labelled mAbs) (BD Biosciences) or with the secondary antibody alone (for the non-conjugated anti-CXCR4 mAbs 44708.111 and 44716.111).

2.4. HIV infection assays

The U87.CD4.CXCR4 transfectants were seeded in 24-well plates (2×10^4 cells per well) and 1000 pg/ml p24 antigen (Ag) of the different HIV-1 strains was added. The cytopathic effect of virus replication in the cell cultures (syncytium formation) was evaluated microscopically at day 5 after infection. Also, cell culture supernatants were collected at day 5 and the HIV-1 core Ag p24 was quantified by enzyme-linked immunosorbent assay (ELISA) (Perkin-Elmer, Boston, MA, USA).

3. Results and discussion

3.1. Expression of the different CXCR4 mutants in stably transfected cell lines

Stably transfected human astroglioma U87.CD4 cell lines, expressing the different CXCR4 mutants (i.e. CXCR4[WT], CXCR4[D171N], CXCR4[D262N], CXCR4[D171N,D262N], CXCR4[D182N], CXCR4[D193N]), were established as described previously [16]. Comparable CXCR4 mRNA expression in the different U87.CD4.CXCR4 transfectants was ascertained by semi-quantitative reverse transcription polymerase chain reaction (data not shown). Adequate and comparable cell surface CXCR4 expression in the different cell lines was demonstrated by flow cytometry after staining with the CXCR4-specific mAb 2B11. This antibody recognizes an epitope in the amino-terminal domain of human CXCR4 [24], which is not affected by the mutations studied here. The mean fluorescence intensities (MFI) for CXCR4-specific staining were 41, 26, 58, 40, 52 and 39 for U87.CD4.CXCR4[WT], U87.CD4.CXCR4[D171N], U87.CD4.CXCR4[D262N], U87.CD4.CXCR4[D171N,D262N], U87.CD4.CXCR4[D182N]

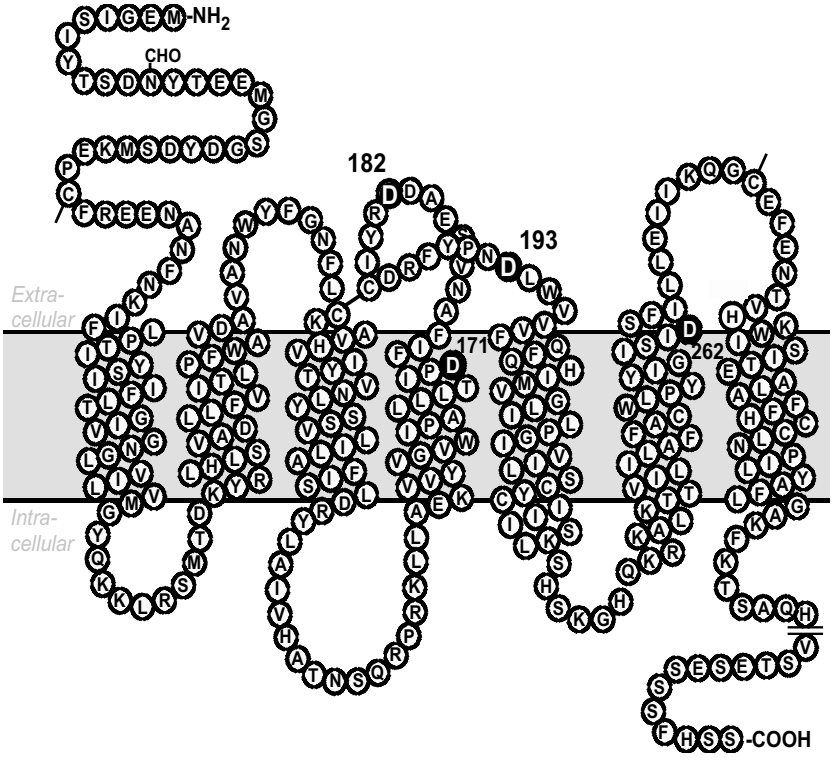

Fig. 1. Amino acid sequence and membrane organization of the chemokine receptor CXCR4 with indication of the positions of the mutated aspartate residues.

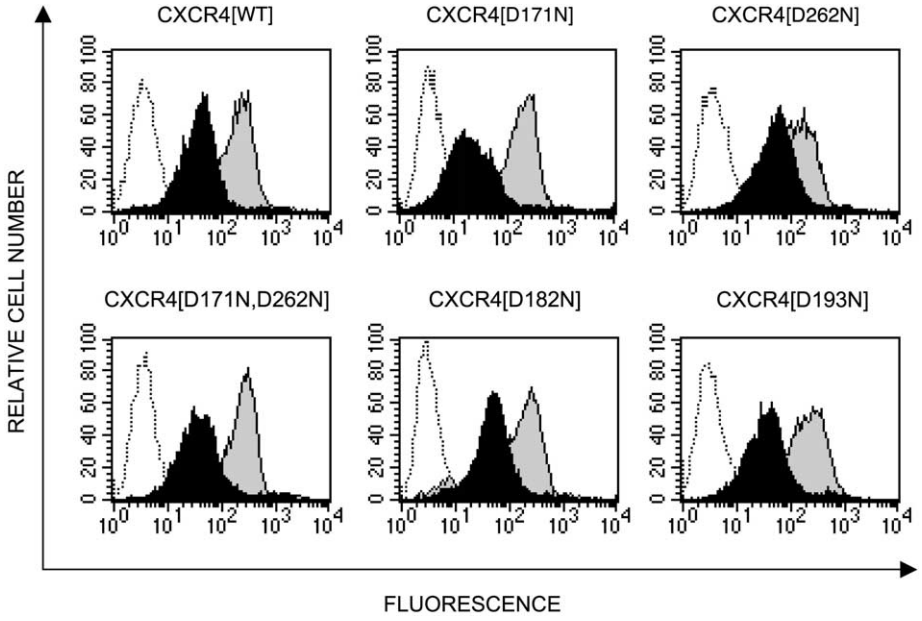

Fig. 2. Flow cytometric analysis of the membrane expression of CD4 and CXCR4 in the stably transfected cell lines. The black histograms represent CXCR4-specific staining by the 2B11 mAb, reacting with an epitope located in the amino-terminus of the receptor protein. The gray histograms represent CD4-specific staining by the SK3 mAb. The white histograms represent the background fluorescence due to non-specific binding of an isotypic control mAb. MFI values are presented in the text.

and U87.CD4.CXCR4[D193N] cells, respectively (Fig. 2, black histograms). The MFI values for non-specific background staining ranged between 3.6 and 4.7 (Fig. 2, white histograms). Abundant cell surface expression of CD4, the primary HIV receptor, was also confirmed by staining of the different U87.CD4.CXCR4 transfectants with anti-human CD4 mAb SK3, yielding MFI values between 144 and 260 (Fig. 2, gray histograms).

3.2. *Effect of aspartate-to-asparagine mutation on antibody recognition of CXCR4*

HIV infectivity depends on the presence of CD4 and an appropriate coreceptor. In addition, virus entry can be influenced by receptor concentration and potentially receptor conformation. It has indeed been shown that 7-TM receptors, like CCR5 and CXCR4, can exist in antigenically distinct conformations on certain cell types [25–27]. As a result, antibodies targeted at conformation-dependent epitopes, such as the most commonly used anti-CXCR4 mAb clone 12G5 [28], may only recognize a subset of CXCR4 molecules expressed at the cell surface [25]. Such conformational heterogeneity may be of great importance in the context of studies examining the molecular interactions between HIV-1 Env and the chemokine receptor. Therefore, we found it useful to evaluate the antibody binding capacities of the different CXCR4 mutants using a panel of four CXCR4-specific mAbs, i.e. the clones 12G5, 44708.111, 44716.111 and 44717.111. These four antibodies are targeted at slightly different epitopes within the second extracellular loop (ECL2) of the receptor protein, which is the area near the amino acid substitution sites (Fig. 1). In contrast with the amino-terminal 2B11 mAb, which showed comparable CXCR4 binding in all six transfectants (Fig. 2, black histograms), marked differences between the CXCR4 variants were observed with the ECL2 binding antibodies (Fig. 3). The CXCR4[D171N,D262N] transfectant showed the lowest binding capacity for each of the four distinct antibodies, while antibody reactivity was partly decreased in the CXCR4[D171N] transfectant. The D262N single mutation had no influence at all. The effect of the D193N substitution was most pronounced with the 12G5 and 44717.111 mAbs, the decrease in 12G5 mAb binding capacity being comparable to that observed in the CXCR4[D171N,D262N] transfectant. The CXCR4[D182N] variant bound the mAbs 44708.111 and 44716.111 with equal efficiency as the wild-type, but showed a slightly reduced affinity for the 12G5 and 44717.111 mAbs (Fig. 3).

3.3. *Coreceptor efficiency of the CXCR4 mutants for diverse HIV-1 strains*

To find out whether the aspartate-to-asparagine substitutions at positions 171, 262, 182 and 193 have any influence on the capacity of CXCR4 to mediate HIV-1 entry, the different CXCR4 transfectants were infected with the X4 HIV-1 strains NL4.3, IIIB and NDK, the dual-tropic R5/X4 HIV-1 strain HE, and the R5/X4 HIV-1 clinical isolate CI-10. The virus-induced cytopathic effect (syncytium formation) on the cell cultures and the virus production, as measured by the p24 core Ag, were evaluated at day 5 after infection.

For HIV-1 NL4.3, p24 viral Ag productions varied $\leq$2-fold among the CXCR4[WT], CXCR4[D171N], CXCR4-[D182N] and CXCR4[D193N] transfectants within each individual experiment. In contrast, NL4.3 virus replication was severely impaired in the U87.CD4.CXCR4[D262N] cells, the p24 Ag production being consistently $\geq$15-fold lower than in the wild-type CXCR4 transfectant (Fig. 4). In the NL4.3-infected U87.CD4.CXCR4[D171N,D262N] cell culture, the p24 Ag concentration measured at day 5 did not exceed the initial virus inoculum. Comparable observations were made with the HIV-1 IIIB strain (Fig. 4). In accord with the p24 Ag measurements, strong cytopathic effects could be microscopically observed at day 5 in the NL4.3- and IIIB-infected CXCR4-[WT], CXCR4[D171N], CXCR4[D182N] and CXCR4-

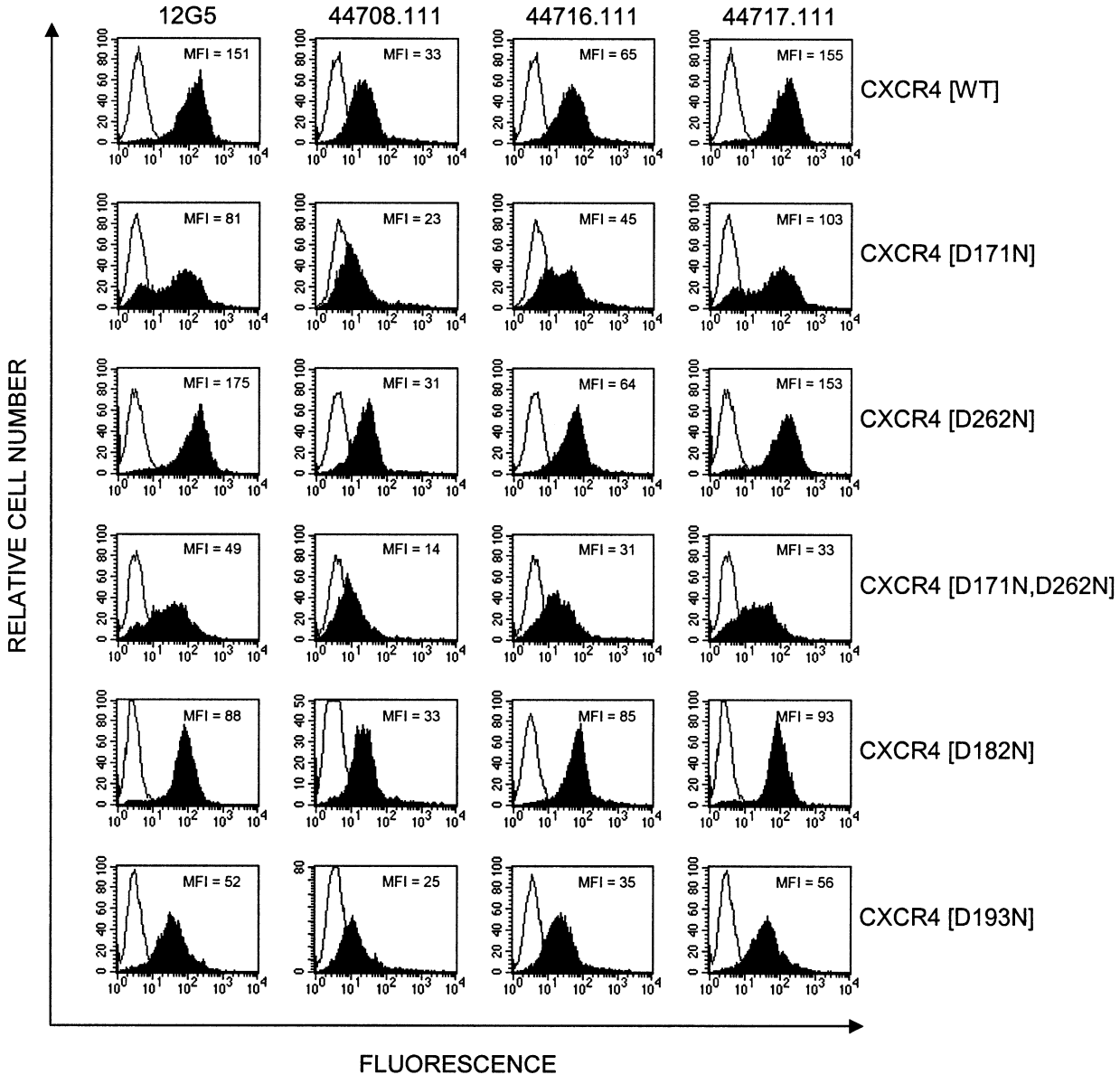

Fig. 3. Staining of the mutant CXCR4 transfectants with four different CXCR4-specific mAbs, reacting with distinct epitopes located in ECL2. The black curves represent CXCR4-specific staining by the 12G5 mAb (left column), by the 44708.111 mAb (second column), by the 44716.111 mAb (third column), or by the 44717.111 mAb (right column). The MFI values for CXCR4-specific staining are indicated in each histogram. The white histograms represent the background fluorescence due to non-specific binding of the secondary antibody (in the case of the 44708.111 and 44716.111 mAbs) or an irrelevant isotypic control mAb (in the case of the 12G5 and 44717.111 mAbs).

[D193N] transfectants, while the extent of giant cell formation was considerably reduced for both viruses in the U87.CD4.CXCR4[D262N] cells, and no visible signs of virus infection could be observed in U87.CD4.CXCR4-[D171N,D262N] cells (data not shown). In accord with these findings, CXCR4 structure–function studies by Brelot et al. also pointed to the critical importance of Asp^{262} for the HIV co-receptor activity of CXCR4 [29].

The D171N,D262N double mutation also seriously limited the coreceptor efficiency of CXCR4 for the R5/X4 HIV-1 clinical isolate CI-10; the CXCR4[D171N,D262N] transfectant yielded a 25-fold lower p24 Ag level than the CXCR4-[WT]-transfected cell line. However, unlike HIV-1 NL4.3 and IIIB, the clinical isolate CI-10 replicated equally well in U87.CD4.CXCR4[D262N] cells as in U87.CD4.CXCR4[WT] cells, but slightly (~3-fold) less efficiently in U87.CD4.CXCR4[D171N] cells (Fig. 4).

A totally different pattern of viral replication was seen with the X4 HIV-1 NDK strain [19]. This virus replicated with comparably high efficiency in the CXCR4[WT], CXCR4-[D171N], CXCR4[D262N], CXCR4[D171N,D262N] and CXCR4[D193N] transfectants, but was strongly affected by the D182N mutation, which caused a 20–40-fold reduction in p24 Ag production (Fig. 4).

On the other hand, none of the mutations negatively influenced the coreceptor function of CXCR4 for the R5/X4 HIV-1 strain HE. After 5 days of infection with the HE strain, no significant reduction in viral replication could be observed

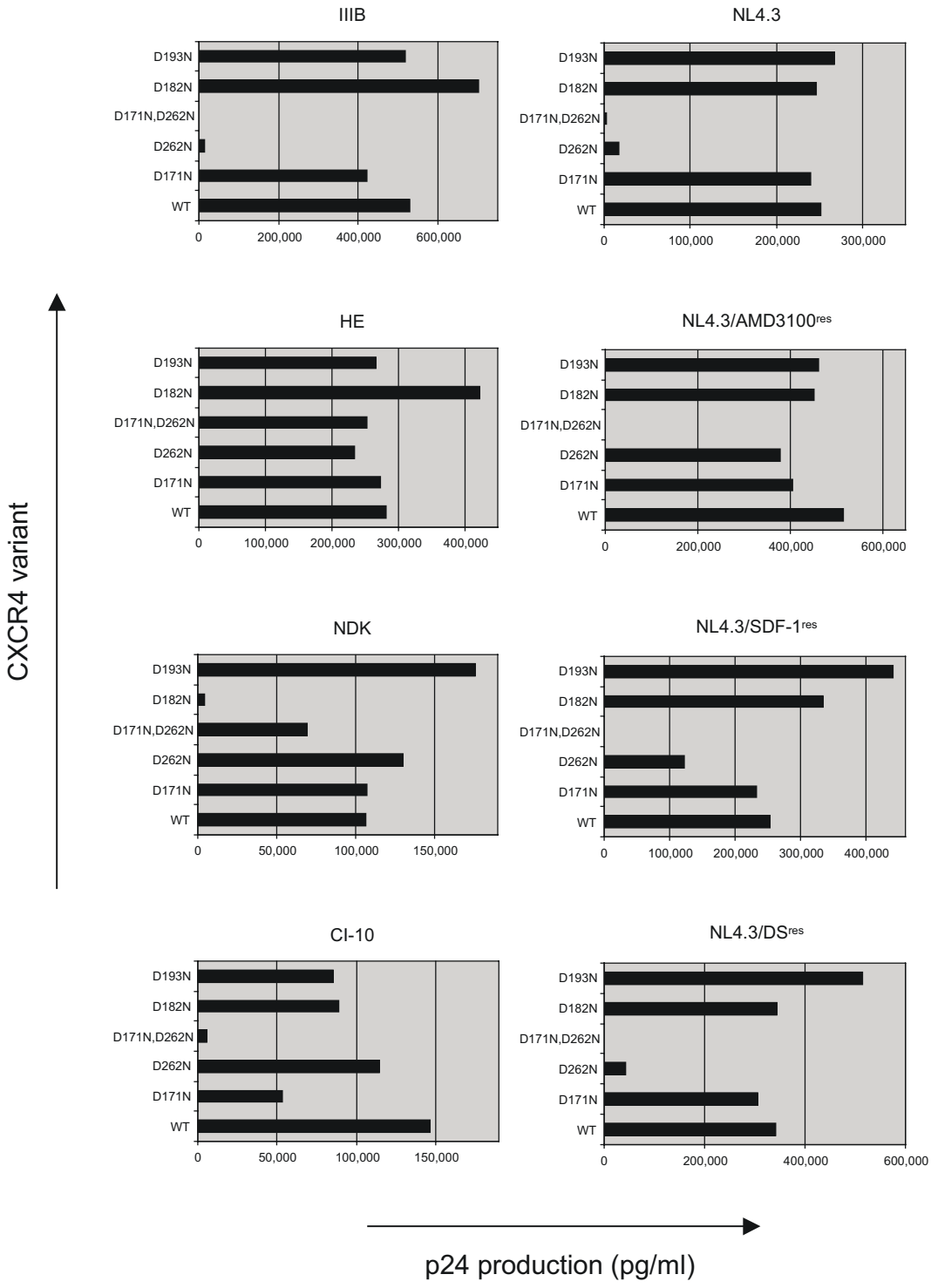

Fig. 4. Viral p24 Ag concentrations measured by ELISA in the culture supernatants of the mutant CXCR4 transfectants at day 5 after infection with the different HIV-1 strains. The data shown are from one representative experiment, which was repeated several times with comparable results.

either microscopically (data not shown) or by p24 Ag measurements (Fig. 4), in any of the mutant CXCR4 transfectants.

Interestingly, Wang et al. reported that conversion of one single aspartate residue of CXCR4, i.e. Asp^{187}, to a neutral amino acid surprisingly unmasks reactivity with R5 viruses, indicating that subtle changes in CXCR4 can dramatically alter utilization by envelopes of varying tropism [30]. However, none of the CXCR4 mutants included in our study supported entry of the R5 HIV-1 strains BaL or ADA (data not shown).

Our data indicate that the envelope gp120 glycoproteins of distinct CXCR4-using HIV-1 strains and clinical isolates largely differ in their dependence on specific amino acid residues of CXCR4 for interaction with the chemokine receptor and subsequent virus entry into the cells. Such diversity

among HIV strains in their structural requirements for functional interaction with CXCR4 was previously reported by Brelot et al. [29,31]. Also, Doranz et al. found that amino acid residues in all four extracellular regions of CXCR4 contribute to coreceptor activity, implying that the viral envelope interacts with a conformationally complex structure of the receptor protein [32]. Yet, AMD3100 is potently and consistently active in the nanomolar range against every CXCR4-using HIV-1 strain tested so far, including those viruses that for their infectivity do not depend on the presence of aspartic acid residues at positions 171 and/or 262 of the coreceptor (e.g. NDK, HE, CI-10). This suggests that the inhibition of virus entry by AMD3100 is not simply due to blockade of a few individual amino acid residues. Presumably, AMD3100 holds the receptor protein in a rigid conformation that does not allow the flexibility and structural rearrangements required for the productive interaction of gp120 with CXCR4 and the subsequent virus–cell fusion process.

3.4. Coreceptor efficiency of the CXCR4 mutants for NL4.3 strains resistant to AMD3100, SDF-1 or dextran sulfate

Given the importance of Asp^{262} for both AMD3100 and HIV-1 NL4.3 gp120 to interact with CXCR4, the question arises whether or not an AMD3100-resistant variant of NL4.3 would retain its dependence on this particular aspartate residue. We have previously selected different NL4.3 strains resistant to either AMD3100 (NL4.3/AMD3100^{res}) [21], SDF-1 (NL4.3/SDF-1res) [22] or the virus binding inhibitor dextran sulfate (NL4.3/DS^{res}) [23]. In MT-4 cells, NL4.3/AMD3100^{res} was ~100-fold less sensitive to AMD3100, and NL4.3/SDF-1res was ~20-fold less sensitive to SDF-1 than the wild-type virus. In addition, NL4.3/AMD3100^{res} showed partial cross-resistance to SDF-1 [33], and, vice versa, NL4.3/SDF-1res also proved 10-fold less sensitive to AMD3100 than the wild-type [22]. Importantly, like wild-type NL4.3, NL4.3/AMD3100^{res} was totally unable to use CCR5 as a coreceptor (data not shown), indicating that resistance to AMD3100 is not associated with a coreceptor switch to CCR5. Likewise, Trkola et al. reported that a primary R5 isolate selected for resistance against a CCR5-specific small molecule inhibitor remained totally dependent on CCR5 and failed to use CXCR4 or any alternative coreceptor for cellular entry [34]. In contrast however, Mosier et al. have found that the RANTES analogue, *N*-nonanoyl(NNY)-RANTES[2–68], potently inhibits R5 HIV-1 replication in vivo in the human peripheral blood lymphocyte-SCID mouse model, but under certain experimental conditions rapidly selects for CXCR4-using mutant viruses [35]. This observation underscores the necessity to simultaneously block all significant viral coreceptors for an effective anti-HIV therapy.

We have now examined the coreceptor efficiency of the different CXCR4 mutants for NL4.3/AMD3100^{res}, NL4.3/SDF-1res and NL4.3/DS^{res}, as compared to wild-type NL4.3. As shown in Fig. 4, AMD3100-resistant NL4.3 clearly acquired the ability to use CXCR4[D262N] as a coreceptor, whereas CXCR4[D171N,D262N] still failed to support virus entry. Moreover, the D262N mutant also gained coreceptor efficiency for the SDF-1-resistant virus, as compared to wild-type NL4.3. This is in agreement with the partial cross-resistance of NL4.3/SDF-1res against AMD3100. In contrast, the NL4.3 strain selected for resistance to dextran sulfate, which does not specifically interact with CXCR4 but blocks virus adsorption to the cell membrane through interaction with the positive charges on the viral gp120 [36], rather behaved like the wild-type virus in our panel of CXCR4 mutants (Fig. 4). Thus, resistance of HIV-1 NL4.3 to AMD3100 is associated with a decreased dependence on Asp^{262}, suggesting a different mode of interaction of the AMD3100-resistant versus wild-type NL4.3 gp120 with CXCR4. One possible explanation could be that the envelope of the AMD3100-resistant NL4.3 strain interacts with a different region of CXCR4. However, as mentioned above, viruses that do not depend on Asp^{262} of CXCR4 for their infectivity can still be blocked by AMD3100, presumably through the conformational restraints imposed on the receptor protein upon binding of the bicyclam. Therefore, we assume that the decreased susceptibility of NL4.3/AMD3100^{res} to AMD3100 results from an increased ability/affinity of the viral gp120 to interact with the AMD3100-bound state of CXCR4. In this respect, it should be noted that resistance to AMD3100 was only obtained after a long-term selection process (>60 passages), remained only partial (~100-fold) and is based on an accumulation of mutations in the gp120 glycoprotein, predominantly clustered in the V3 loop [21]. In comparison, for certain anti-HIV drugs, such as the non-nucleoside reverse transcriptase inhibitor TSAO, >1000-fold resistance resulting from a single point mutation in the viral target enzyme can be achieved within 2–3 weeks [37].

3.5. Conclusion

In summary, we found that mutation of Asp^{262} of CXCR4 does not affect antibody recognition of the receptor molecule but has a large impact on its coreceptor function for HIV-1 viruses. Furthermore, our data point to the wide variation among different viruses in their mode of interaction with the chemokine receptor. Yet, the high-affinity binding of the bicyclam AMD3100 to Asp^{171} and Asp^{262} of CXCR4 has conformational consequences that affect the HIV-1 coreceptor function of CXCR4 in general, as X4 viruses that do not require Asp^{262} as a CXCR4 interaction site are still effectively blocked by AMD3100. Apparently, the structural limitations imposed on CXCR4 upon binding of AMD3100 cannot easily be overcome by the emergence of resistant virus.

Acknowledgements: This work was supported by grants from the Fonds voor Wetenschappelijk Onderzoek (FWO)-Vlaanderen (Krediet G.0104.98), and the Geconcerteerde Onderzoeksacties (Vlaamse Gemeenschap) (Krediet 00/12). S.H. is a Postdoctoral Research Assistant of the Fonds voor Wetenschappelijk Onderzoek (FWO)-Vlaanderen. We are grateful to Sandra Claes and Eric Fonteyn for their excellent technical assistance.

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