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Conjugation of Soluble CD4 without Loss of Biological Activity via a Novel Carbohydrate-directed Cross-linking Reagent*

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Chemical conjugates of recombinant soluble CD4 (sCD4) with toxins, or with antibodies that activate cytotoxic T cells, can be used to direct selective killing of human immunodeficiency virus (HIV)-infected cells. This approach takes advantage of the ability of sCD4 to bind with high affinity to gp120, the envelope protein of HIV-1, which is expressed on actively infected cells. However, conjugation of sCD4 via reagents that target amino groups may reduce its affinity for gp120, since at least one such group is important for gp120 binding. Here, we describe a novel cross-linking reagent which enables the conjugation of sCD4 via its carbohydrate moieties rather than its free amino groups. This heterobifunctional reagent, 4-(4-*N*-maleimidophenyl)butyric acid hydrazide (MPBH), combines a nucleophilic hydrazide with an electrophilic maleimide, thereby allowing coupling of carbohydrate-derived aldehydes to free thiols. We describe conditions by which MPBH is coupled selectively to the sialic acid residues of sCD4, and exemplify the use of MPBH by conjugating sCD4 to hemoglobin and to β-galactosidase. We show that, whereas conjugation of sCD4 via amino groups markedly reduces its gp120 binding affinity, conjugation via the carbohydrate chains using MPBH does not affect binding. Moreover, we demonstrate the ability of a sCD4-MPBH-fluorescein conjugate to label HIV-infected human CEM cells selectively. These results indicate that, by targeting its carbohydrate moieties, sCD4 can be cross-linked to other molecules without compromising its function. The approach described here can be useful for glycoproteins in which amino groups, but not carbohydrates, are important for function. More generally, this approach can be considered for use in cross-linking glycoconjugates to compounds which either contain thiols, or to which thiols can be added.

CD4 is a transmembrane glycoprotein, found on the surface of human T lymphocytes, that acts as the primary receptor for HIV-1[1] (1, 2). The extracellular portion of CD4 contains four immunoglobulin-like domains (V_1–V_4), the first of which (V_1) is necessary and sufficient for high affinity binding to gp120, the envelope glycoprotein of HIV-1 (3–5). We (6) and others (7–11) have produced a recombinant, soluble form of CD4 (sCD4), containing only the extracellular portion of the molecule. sCD4 retains high affinity binding to gp120 and can block the binding of HIV-1 to cell surface CD4 *in vitro*, thereby inhibiting infection of target cells (reviewed in Ref. 12).

Several modifications of sCD4 have been made, by either gene fusion or chemical conjugation approaches, to expand its antiviral capabilities. One example is a class of chimeric molecules known as CD4 immunoadhesins, in which genes encoding sCD4 and immunoglobulin G heavy chain are combined, thus creating homodimeric antibody-like molecules with properties of both CD4 and human immunoglobulin (13–15). Other notable examples of modified sCD4 are CD4 peptide-protein conjugates (16), CD4 electroinserted into erythrocyte membranes (17), and sCD4-toxin hybrids, in which sCD4 is attached to a toxin either by gene fusion (18, 19) or by chemical cross-linking (20); because cells infected actively with HIV express gp120 on their surface, sCD4 can be used to direct toxins to, and thus selectively kill, HIV-infected cells. Similarly, bispecific hybrids of sCD4 and anti-CD3 antibody can be constructed which mediate selective killing of HIV-infected cells by cytotoxic T cells (21, 22). These examples represent some of the possible modifications of sCD4 which may improve its efficacy against HIV *in vivo*.

In the present work we have focused on developing a new approach for chemical conjugation of sCD4. In our attempts to derivatize sCD4 using conventional cross-linking reagents that attach to amino groups, we found that binding of the derivatized receptor to HIV-1 gp120 was affected adversely. This was not surprising, since there is at least 1 lysine residue in sCD4 which is required for high affinity binding to gp120 (reviewed in Refs. 23 and 24). Given that (i) residues within the V_1 domain of sCD4 are principally responsible for gp120 binding and (ii) sCD4 is glycosylated in the V_3, but not the V_1 domain (25–27), we reasoned that conjugating sCD4 via its carbohydrate moieties might not affect gp120 binding. A similar prediction was made by Rodwell *et al.* (28), who

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[1] The abbreviations used are: HIV, human immunodeficiency virus; β-gal, β-galactosidase; BSA, bovine serum albumin; Hb, hemoglobin; MPBH, 4-(4-*N*-maleimidophenyl)butyric acid hydrazide; PBS, phosphate-buffered saline; SAMSA, 5-((2(and-3-)-*S*-(acetylmercapto)-succinoyl)amino); sCD4, recombinant soluble CD4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMPB, *N*-succinimidyl-4-(*p*-maleimidophenyl)butyrate; FACS, fluorescence-activated cell sorting.

showed that immunoglobulins, modified via oligosaccharide moieties, retained fully their antigen binding affinities.

Therefore, we designed and synthesized a cross-linking agent which enables the conjugation of sCD4 by reacting with its carbohydrate portion. This reagent, 4-(4-*N*-maleimidophenyl)butyric acid hydrazide (MPBH),[2] generates a stable conjugate by cross-linking carbohydrate-derived aldehydes of a glycoprotein, such as sCD4, to thiol-containing compounds. We show that sCD4 conjugated with MPBH retains its high binding affinity to gp120 and is capable of labeling HIV-infected cells selectively.

MATERIALS AND METHODS

Reagents—Human hemoglobin (Hb) was from Sigma, *Escherichia coli* β-galactosidase (β-gal) (no. 567–779; 600–950 units/mg protein) and 2-nitrophenyl-β-D-galactoside were from Boehringer Mannheim (Indianapolis, IN), recombinant sCD4 (6, 26) and CD4-IgG (13) were purified and characterized at Genentech, SMPB was from Pierce Chemical Co. (Rockford, IL), and 5-((2(and-3-)-*S*-(acetylmercapto)succinoyl)amino)fluorescein (SAMSA fluorescein, A-685) was from Molecular Probes (Eugene, OR). Reagents for synthesis of MPBH were from Sigma or Aldrich and were used without further purification. All other chemicals were of analytical grade.

Synthesis and Characterization of MPBH—Reactions were performed under an inert atmosphere of nitrogen or argon. Spectra were recorded on Nicolet 510 FT-IR (infrared), Varian VXR-300 S (300 MHz) (NMR), and Jeol JMS-HX110HF (mass spectra) spectrometers.

4-Aminophenylbutyric acid (2.0 g, 11.2 mmol) and 2,6-lutidine (1.30 ml, 11.2 mmol) were warmed in tetrahydrofuran (40 ml) to give a homogeneous solution, and were added dropwise over 5 min to a solution of maleic anhydride (1.09 g, 11.2 mmol) in ether (20 ml), heated at reflux. Heating was continued for 30 min and the product was collected by filtration, washed with ether, and vacuum-dried. Acetic anhydride (10 ml) and sodium acetate (0.50 g) were added, and the mixture was heated and stirred at 100 °C (30 min), to obtain a homogeneous brown solution. After cooling, the solution was poured into a mixture of ice and 1 N HCl (50 ml) and extracted with ethyl acetate (2 × 50 ml). The combined extracts were washed sequentially with water and brine, then dried ($MgSO_4$), and evaporated to give the crude maleimide (2.38 g).

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.94 g, 10.1 mmol) was added to a solution of the crude maleimide (2.38 g), *t*-butylcarbazate (1.33 g, 10.1 mmol) and hydroxybenzotriazole (1.36 g, 10.1 mmol) in 30 ml of dimethylacetamide and then stirred at room temperature (18 h). The mixture was diluted with 50 ml of water and extracted with ethyl acetate (2 × 100 ml). The combined extracts were washed sequentially with 1 N HCl, saturated sodium bicarbonate, and brine, and then dried ($MgSO_4$), and the solvent was removed by evaporation. The product was purified using silica gel high performance liquid chromatography. A 21.4-mm (inner diameter) × 25-cm column was loaded and eluted with a gradient of 1:1 EtOAc/hexane to 7:3 EtOAc/hexane over 40 min at a flow rate of 20 ml/min. The product eluted after 14.7 min (detection at 254 nm), to give 4-(4-*N*-maleimidophenyl)butyric acid, *N*-2-*t*-butyloxycarbonylhydrazide (1.51 g, 44%), as a pale yellow oil. ^1H NMR ($CdCl_3$) δ 7.40 (br s, 1H, NH), 7.28 (d, J = 8.6 Hz, 2H, Ar), 7.25 (d, J = 8.6 Hz, 2H, Ar), 6.85 (s, 2H, CH = CH), 6.55 (br s, 1H, NH), 2.71 (t, J = 7.6 Hz, 2H, CH_2Ph), 2.23 (t, J = 7.4 Hz, 2H, CH_2CO), 2.02 (m, 2H, CH_2), 1.47 (s, 9H, Me_3); IR (neat) 3295, 2979, 1714, 1516, 1396, 1244, 1155, 828 (s), 691 (s) cm^{-1}; MS (FAB) *m/e* (relative abundance) 374 (MH^+, 100).

$C_{19}H_{23}N_3O_5$

Calculated: C 61.1 H 6.2 N 11.3

Found: C 60.8 H 6.05 N 11.2

4-(4-*N*-Maleimidophenyl)butyric acid, *N*-2-*t*-butyloxycarbonylhydrazide (1.50 g, 4.02 mmol) was dissolved in 4 N HCl in dioxane (10 ml) and stirred at room temperature (30 min). Excess HCl was removed by degassing with argon, and the solvent by evaporation. The residue was triturated with ether (3×) and dried under vacuum to give 4-(4-*N*-maleimidophenyl)butyric acid, hydrazide, hydrochloride (1.02 g, 82%), as a pale yellow powder. ^1H NMR (d_6-dimethyl sulfoxide) δ 10.4 (br s, 2H, NH_2), 7.31 (d, J = 8.3 Hz, 2H, Ar), 7.25 (d, J = 8.3 Hz, 2H, Ar), 7.18 (s, 2H, CH = CH), 3.45 (br s, 2H, NH_2), 2.64 (t, J = 7.5 Hz, 2H, CH_2Ph), 2.27 (t, J = 7.3 Hz, 2H, CH_2CO), 1.87 (m, 2H, CH_2); IR (KBr) 2940, 1708, 1516 (s), 1401, 1151, 823 (s), 690 (s) cm^{-1}; MS (FAB) *m/e* (relative abundance) 274 (MH^+, 100).

$C_{14}H_{15}N_3O_3 \cdot HCl$

Calculated: C 54.3 H 5.2 N 13.6

Found: C 54.1 H 5.1 N 13.3

The final product was determined to be stable for several months if stored dry at 2–8 °C and was freshly dissolved in *N,N*-dimethylformamide for use.

Oxidation of sCD4 and Coupling to MPBH—Two sets of conditions were used for oxidation of the carbohydrate portion of sCD4. sCD4 (10 mg/ml) was oxidized in 0.1 M NaOAc, pH 5.5, containing either 1 mM $NaIO_4$ (0.5–3 h, 0 °C) (29) or 10 mM $NaIO_4$ (0.5–3 h, 23 °C) (30).[3] Residual $NaIO_4$ was removed from the oxidized glycoprotein by gel filtration on Sephadex G25 equilibrated in 0.1 M NaOAc, pH 5.5. To oxidized sCD4 (1–4 mg/ml) was added MPBH (10 mg/ml in *N,N*-dimethylformamide) to a final concentration of 1 mM; conjugation of MPBH to sCD4 proceeded for 0–3 h at 23 °C. For gp120 binding studies, the buffer was first exchanged to 0.1 M $NaPO_4$, pH 7.0, 50 mM NaCl; then sCD4-MPBH was reacted with 5 mM 2-mercaptoethanol for 40 min on ice (converting its maleimide moiety to a thioether), then recovered again by gel filtration in the same pH 7.0 buffer. Alternatively, to identify monosaccharides which were oxidized under the two sets of conditions, oxidized sCD4 was recovered free of residual $NaIO_4$ and analyzed for carbohydrate content (26).

Kinetics of MPBH Hydrazide Coupling to Oxidized sCD4—For kinetic measurements, sCD4-MPBH was recovered by gel filtration on Sephadex G25 equilibrated in 10 mM NaOAc, pH 5.5, 150 mM NaCl. To sCD4-MPBH (0.5 ml) was added 4 mCi of [^{35}S]cysteine (5.5 GBq/mmol, Amersham) in 0.2 M $NaPO_4$, pH 7.0 (0.5 ml); after 15 min at 23 °C, ^{35}S-labeled glycoprotein was recovered again by gel filtration. To determine specific radioactivity as a means of quantitating the molar ratio of MPBH:sCD4, duplicate aliquots were assayed for radioactivity and for protein concentration (31).

pH Optimization of MPBH Maleimide Reaction—MPBH (230 μM prepared from a 10 mM stock solution in *N,N*-dimethylformamide) in 0.1 M sodium acetate, pH 5.5, or 0.1 M $NaPO_4$, pH 7.0, was incubated with 100 μM dithiothreitol (5 min, 23 °C), and reaction of the thiol with maleimide to form the thioether was followed by loss of absorbance at 320 nm ($\epsilon_{320\,nm}$ = 35.1).[4] Hydrolysis of the maleimide of MPBH was measured by incubating MPBH (230 μM) at pH 5.5 or 7.0 (as above), or in 0.1 M sodium borate, pH 8.0, and omitting dithiothreitol. Base-catalyzed conversion of the maleimide to maleamic acid (32) was followed also at 320 nm, but in this case, by an increase in absorbance.

Conjugation of sCD4-MPBH to Thiol-containing Proteins—Human Hb or *E. coli* β-gal (each 0.4 ml, 5 mg/ml) were coupled to sCD4-MPBH (2 ml, 4 mg/ml), prepared as described above. Coupling was performed in 0.1 M $NaPO_4$, pH 7.0 for 2 h at 23 °C. β-Gal activity was assayed before and after conjugation using 2-nitrophenyl-β-D-galactoside as substrate. Conjugates were analyzed by SDS-PAGE (9% for sCD4-Hb; 7.5% for sCD4-β-gal) (33) under reducing conditions and detected by staining with Coomassie Blue. For gp120 binding studies, sCD4-β-gal was purified free of residual sCD4 by fast protein liquid chromatography size exclusion chromatography on Superose 6 (1 × 30 cm) equilibrated in 0.1 M sodium phosphate, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Tween 20.

Conjugation of sCD4-MPBH to SAMSA Fluorescein—sCD4-MPBH was coupled to SAMSA fluorescein as follows; 2.6 mg of sCD4-MPBH (63 nmol, prepared using the mild oxidation conditions described above), in 1.3 ml of 0.1 M $NaPO_4$, pH 7.0, was mixed with 10 mg of activated SAMSA fluorescein (19 μmol in 1.2 ml of 0.1 M

[2] The heterobifunctional reagents *N*-succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB) and MPBH contain the same 4-phenylbutanoyl spacer.

[3] Throughout the text, the former set of conditions is referred to as "mild" oxidation and the latter as "harsh" oxidation.

[4] MPBH absorbs ultraviolet light in a spectrum characteristic of its phenylmaleimide chromophore. Its λ_{max} is actually 305 nm; however, we chose to detect it at 320 nm to avoid interference from protein absorbance.

$NaPO_4$, pH 7.0; see below) in a final volume of 2.5 ml. After 30 min at room temperature, sCD4-MPBH-fluorescein (2.6 mg, 0.65 mg/ml) was recovered by gel filtration on Sephadex G25 equilibrated in PBS. SAMSA fluorescein was activated for use as recommended by the manufacturer; 10 mg of SAMSA fluorescein was dissolved in 1 ml of 0.1 N NaOH and incubated at room temperature for 15 min to remove the thiol protecting group. The reagent solution was neutralized (14 μl of 6 N HCl), and buffered by the addition of 0.2 ml of 0.5 M $NaPO_4$, pH 7.0, to give a final reagent solution of 10 mg/1.2 ml, which was used immediately.

Coupling of SMPB to sCD4—sCD4 (3.6 mg/ml) in 0.1 M $NaPO_4$, pH 7.0, 50 mM NaCl was reacted with 1 mM SMPB (10 mg/ml in dimethyl sulfoxide) for 1 h at 23 °C. As was done for sCD4-MPBH, this step was followed by incubation of sCD4-SMPB for 15 min in 5 mM 2-mercaptoethanol, then recovery by gel filtration in 0.1 M $NaPO_4$, pH 7.0, 50 mM NaCl.

gp120 Binding—The ability of sCD4 conjugates to bind to gp120 was determined as described previously (34, 35). Briefly, CD4-IgG (13) was immobilized onto microtiter wells with anti-IgG antibody. Then, sCD4 or its derivative was added simultaneously with ^{125}I-labeled gp120 ($HIV\text{-}1_{IIIB}$) to determine the ability of sCD4 (or derivatized sCD4) to compete with the CD4-IgG for gp120 binding. The assays were done in triplicate; nonspecific binding was determined by omitting CD4-IgG.

Labeling CEM Cells with sCD4-MPBH-Fluorescein—The CEM.NKR T-lymphoblastoid cell line (36) was infected with $HIV\text{-}1_{IIIB}$ as described (37). One million viable, HIV-1-infected cells or uninfected cells were incubated in 100 μl of sCD4-MPBH-fluorescein (1 μg/ml) in Dulbecco's PBS (GIBCO) containing 2% BSA for 30 min on ice. Control incubations of HIV-1-infected or uninfected cells were performed in PBS, 2% BSA (no sCD4-MPBH-fluorescein) under the same conditions. The cells were washed twice with 50 volumes of cold PBS, 2% BSA mixture and fixed by incubation in 2% paraformaldehyde in PBS for 24 h at 4 °C. Cell fluorescence was measured using a FACStar flow cytometer and LYSIS-II software (Becton-Dickinson, Mountain View, CA).

RESULTS

Design of MPBH—MPBH was designed to cross-link the oligosaccharides of sCD4 to thiol-containing compounds. The reagent (Fig. 1) consists of two reactive moieties, a hydrazide and a maleimide, separated by a 4-phenylbutanoyl spacer. The reaction to form a macromolecular conjugate via MPBH is a three-step process: (i) oxidation of vicinal diols in the glycoprotein to generate aldehydes in the oligosaccharide portion, (ii) reaction of the aldehydes with the hydrazide function of MPBH to form a hydrazone bond between the glycoprotein and the cross-linking reagent, and (iii) reaction of the maleimide function of MPBH to form a thioether bond with a thiol-containing protein, thus creating a stable glycoprotein-protein conjugate.

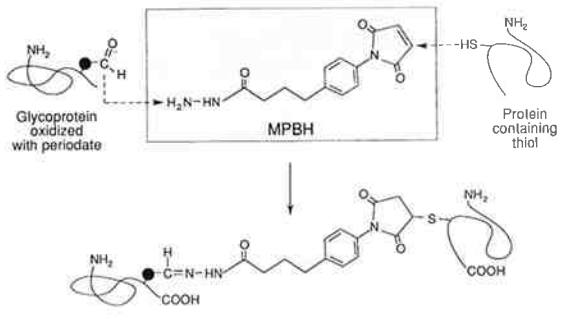

FIG. 1. **Cross-linking with MPBH.** MPBH is heterobifunctional, containing hydrazide and maleimide groups, which react with aldehydes and thiols, respectively. Formation of a conjugate between a glycoprotein and a thiol-containing protein is achieved in three steps: (*a*) oxidation of the carbohydrate chains of the glycoprotein with $NaIO_4$ to generate aldehydes; (*b*) condensation of hydrazide and aldehyde, attaching the MPBH reagent to the glycoprotein via a stable hydrazone; (*c*) attachment of a thiol-containing protein to the maleimide-glycoprotein via a thioether linkage to form the complete conjugate. The glycoprotein may be replaced by any glycoconjugate, and the thiol-containing protein by any thiol-containing compound. To facilitate conjugation via this method, thiols can also be added to compounds which lack them (48–50).

Oxidation and Coupling of sCD4 to MPBH—Recombinant human sCD4, expressed in Chinese hamster ovary cells, contains two Asn-linked oligosaccharide chains at Asn-271 and Asn-300 (25, 26); its carbohydrate composition, on a molar basis, is as follows: fucose, 0.5; *N*-acetylglucosamine, 6.3; galactose, 4.0; mannose, 7.1; and sialic acid, 2.7 (26). To identify conditions that might allow incorporation of different amounts of MPBH into sCD4 by selective oxidation of monosaccharides, we compared the effect of mild *versus* harsh oxidation conditions on the different sugars in sCD4 (Table I). Under the mild conditions (0 °C, 1 mM periodate at pH 5.5), only minor degradation of fucose, *N*-acetylglucosamine, galactose, and mannose residues was observed, whereas sialic acid residues were converted quantitatively to a derivative (probably oxidized at C-7, C-8, and C-9, with loss of C-8 and C-9 as formaldehyde) within 3 h of treatment. Thus, periodate treatment under these conditions appears to oxidize sialic acid residues of sCD4 selectively, consistent with previous work (29). In contrast, under the harsh conditions (room temperature, 10 mM periodate, pH 5.5), a more extensive and less selective oxidation of carbohydrates was observed. This is consistent with previous observations upon oxidation of immunoglobulins (30). Sialic acid and fucose were most affected, followed by mannose, galactose, and *N*-acetylglucosamine, in decreasing order. The concentration of periodate and the temperature of reaction appeared to be the most significant factors in determining the selectivity of oxidation.

We investigated the rate and extent of coupling of MPBH to oxidized sCD4 over a 3-h period by using [^{35}S]cysteine to quantitate the MPBH-maleimides bound to sCD4 (Fig. 2). sCD4 oxidized under mild conditions showed a modest rate of incorporation of MPBH, and, within 3 h, approximately 0.5 mol of MPBH was incorporated per mol of sCD4. In contrast, sCD4 oxidized under harsh conditions showed significantly more rapid incorporation of MPBH, which began to plateau within 3 h at about 1.7 mol/mol. The lower incorporation of MPBH via the mild oxidation conditions is consistent with fewer aldehydes having formed in the carbohydrate moieties of sCD4 under these conditions (Table I).

To develop the coupling conditions further, we characterized the pH dependence of the rate at which a thiol reacts with the maleimide of MPBH. As might be expected for a reaction in which deprotonated thiol is the nucleophilic species, the rate was rapid at pH 7.0 and slower at pH 5.5 (Fig. 3*A*). However, MPBH is susceptible to base-catalyzed hydrolysis which converts the maleimide to maleamic acid (32).

TABLE I

Carbohydrate composition analysis of sCD4 oxidized under two different conditions

sCD4 was treated with $NaIO_4$ under two sets of conditions to generate aldehydes by oxidation of carbohydrate residues. Oxidation conditions were 1 mM $NaIO_4$, 0 °C (mild); or 10 mM $NaIO_4$, 23 °C (harsh). Both treatments were performed in 0.1 NaOAc, pH 5.5 for 3 h. Data are presented as mol of carbohydrate/mol of protein.

Carbohydrate	No oxidation[a]	Mild oxidation	Harsh oxidation
	mol/mol	*mol/mol*	*mol/mol*
Fuc	0.5	0.5	0.1
GlcNAc	6.3	6.2	4.0
Gal	4.0	3.7	1.7
Man	7.1	7.1	2.8
Sia	2.7	0.1	0.0

[a] Data from Harris *et al.* (26).

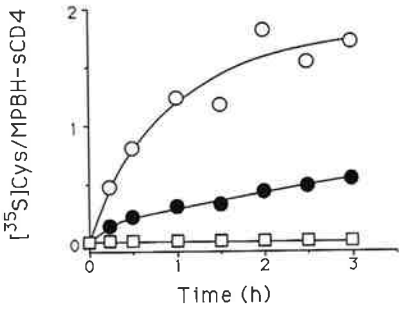

FIG. 2. **Rate and extent of addition of MPBH to sCD4, oxidized under mild or harsh conditions.** sCD4 (1.2 mg/ml), untreated (□), or oxidized under mild (●) or harsh (○) conditions, was incubated at room temperature with 1 mM MPBH in 0.1 M NaOAc, pH 5.5. The reaction was stopped by removing excess reagent, and 4 mCi of [^{35}S]cysteine in 0.1 M $NaPO_4$, pH 7.0 was added. After 15 min, ^{35}S-labeled sCD4 was recovered by gel filtration and duplicate aliquots were assayed for radioactivity and protein concentration. sCD4 was assumed to have M_r = 41,000 (26).

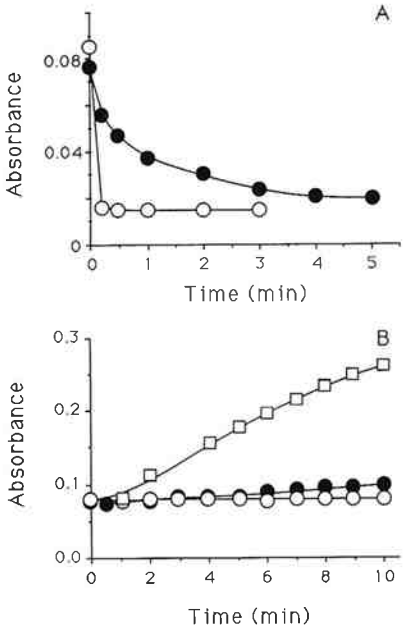

FIG. 3. **pH optimization of MPBH reaction with free thiol.** *Panel A* shows the reaction of MPBH with dithiothreitol. MPBH (230 μM) in 0.1 M buffer was reacted at pH 5.5 (●) or pH 7.0 (○) at room temperature with dithiothreitol (100 μM). The reaction was followed by a decrease in absorbance at 320 nm, resulting from conversion of the maleimide to a thioether. *Panel B* shows the hydrolysis of MPBH as a function of pH. In this case, absorbance at 320 nm increased due to base-catalyzed conversion of the maleimide to maleamic acid, and therefore could be used to monitor the hydrolysis of MPBH. MPBH (230 μM) was incubated in 0.1 M buffer at pH 5.5 (○), pH 7.0 (●), or pH 8.0 (□) at room temperature.

To attempt to minimize this, we tested the rate of hydrolysis at several pH values. The hydrolysis was slow at pH 5.5 and 7.0 and markedly more rapid at pH 8.0 (Fig. 3*B*). Therefore, pH 7.0 appeared to be optimal for thiol addition.

Formation of sCD4-Protein Conjugates—We chose Hb and β-gal as thiol-containing proteins to model the cross-linking ability of MPBH. sCD4 was oxidized under mild or harsh conditions, conjugated via MPBH to Hb or β-gal, and the conjugation products were analyzed by SDS-PAGE (Fig. 4). Hb is a 64-kDa tetramer containing two α and two β subunits associated non-covalently; a single free thiol is present in each of the 16 kDa β-chains. Therefore, sCD4 would be predicted to form a (50 + 16) = 66-kDa covalent species with each Hb

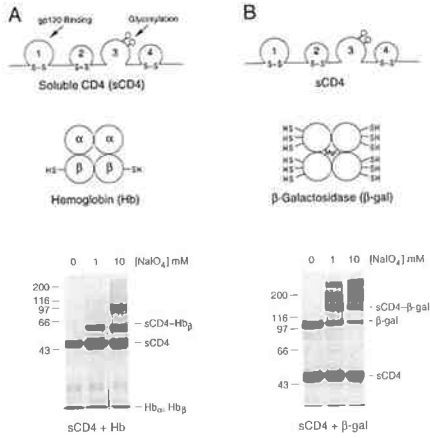

FIG. 4. **Formation of sCD4 conjugates with hemoglobin and β-galactosidase.** The four-domain structure of sCD4 and the subunit structures of Hb and β-gal are shown schematically. Domains of sCD4 labeled *1* and *3* are referred to in the text as "V_1" and "V_3," respectively. sCD4 was oxidized under the mild (1) or harsh (10) conditions (see "Materials and Methods") and reacted with MPBH, then incubated with Hb (*A*) or β-gal (*B*). Samples in which the oxidation step was omitted (*0*) represent polypeptides initially present in the reaction mixture. Bands labeled *sCD4-Hbβ* and *sCD4-β-gal* are monovalent conjugates of 66 and 166 kDa, respectively, while bands of higher molecular mass probably represent conjugates that are poly-addition products. Reaction products were analyzed by 9% or 7.5% SDS-PAGE, respectively.

β-chain. When the oxidation step was omitted, only the separate polypeptides (sCD4, Hb α- and β-chains) were present (Fig. 4*A*). However, with mild oxidation of sCD4, reaction with MPBH and Hb resulted in the appearance of a single new band at 66 kDa, indicating the formation of a monovalent sCD4-Hb_β conjugate. When the harsh oxidation conditions were used, several higher molecular mass bands appeared in addition to the 66-kDa band. These bands probably represent the products of poly-addition of Hb_β to sCD4, since under harsh oxidation conditions, multiple MPBH molecules (and thus multiple Hb_β chains) can be incorporated into each molecule of sCD4 (Fig. 2).

β-Gal is a homotetramer consisting of 116-kDa subunits associated non-covalently; each subunit contains three free thiols. Thus, conjugation of sCD4 to β-gal could produce a variety of products, ranging from 1 to 3 sCD4 molecules/β-gal subunit. Indeed, SDS-PAGE analysis of sCD4-β-gal conjugation products revealed the presence of multiple bands of molecular mass equal to or greater than (50 + 116) = 166 (the sum of M_r of sCD4 plus a β-gal subunit) (Fig. 4*B*). Multiple bands were observed with sCD4 oxidized either under mild or harsh conditions. Notably, mild oxidation of sCD4 resulted in the production of more monovalent sCD4-β-gal conjugate. This is probably due to incorporation of less MPBH into sCD4 under these conditions (Fig. 2), which may limit the extent of the sCD4 + β-gal reaction.

Conjugate formation was not quantitative in either the Hb or β-gal case; approximately 30% of the Hb and 80% of the β-gal were cross-linked to the oxidized sCD4. The enzymatic activity of β-gal conjugated to sCD4, as measured by the ability of the enzyme to hydrolyze the synthetic substrate 2-nitrophenyl-β-D-galactoside, was not significantly different from that of unconjugated β-gal (data not shown). This observation is consistent with previous data showing that modification of β-gal via its free thiols does not affect its enzymatic activity (38). Moreover, it suggests the possibility that conjugation of sCD4 via MPBH to toxins may not affect their

enzymatic activity, and thus not compromise their ability to kill target cells.

Effect of sCD4 Conjugation on gp120 Binding—We investigated whether MPBH-mediated coupling via the carbohydrate moieties of sCD4 affects gp120 binding affinity. For comparison, we also tested the binding of sCD4 coupled to SMPB, a reagent which attaches to free amino groups (39). sCD4, and sCD4 oxidized under mild conditions and conjugated to MPBH, exhibited similar gp120 binding affinities (Fig. 5). This sCD4-MPBH preparation contained a significant fraction of unconjugated sCD4 (under these oxidation and coupling conditions, the ratio of MPBH incorporation was 0.5 mol/mol; see Fig. 2). Nevertheless, the virtual identity of the competition curves suggests that the sCD4-MPBH conjugate in this preparation bound to gp120 as well as sCD4. Furthermore, sCD4 oxidized under harsh conditions and conjugated to MPBH also bound to gp120 with an affinity similar to sCD4. Based on the data presented in Fig. 2, this preparation of sCD4-MPBH contained little or no unconjugated sCD4 (the ratio of MPBH incorporation was 1.7 mol/mol). Therefore, taken together, these results indicate that conjugation of MPBH to sCD4 does not affect gp120 binding.

We tested also the binding of sCD4-β-gal conjugates to gp120. In preparing conjugates for this purpose, we used the mild oxidation conditions in order to obtain a preparation that was enriched in monovalent sCD4-β-gal conjugate. Also, in this case, we removed unconjugated sCD4 by size exclusion chromatography (see "Materials and Methods"). The binding of these purified sCD4-β-gal conjugates to gp120 was similar to that of sCD4 (Fig. 5), demonstrating that actual conjugation of sCD4 to another protein, via MPBH, does not affect its biological activity significantly.

In contrast to the three sCD4-MPBH conjugates described above, which exhibited half-maximal inhibition (IC_{50}) values ranging from 6–9 nM, sCD4 coupled to SMPB exhibited a markedly lower affinity for gp120 (IC_{50} = 27 nM) (Fig. 5). Thus, whereas conjugation of sCD4 via its carbohydrate moieties does not appear to affect gp120 binding, conjugation via free amines reduces the gp120 binding affinity significantly.

sCD4-MPBH-Fluorescein Labels HIV-infected Cells Selectively—To investigate whether a sCD4-MPBH conjugate could be used as a targeting vector that is selective for HIV-1-infected cells, we conjugated sCD4 to a reporter molecule, fluorescein, via MPBH. Binding analysis (Fig. 5) showed that sCD4-MPBH-fluorescein binds gp120 with an affinity comparable with that of unmodified sCD4. Next, we infected human lymphoid CEM cells with HIV-1 (IIIB strain) and tested the ability of sCD4-MPBH-fluorescein to bind to these cells, which remain viable during the assay, by FACS analysis (Fig. 6). Whereas no change in fluorescence was observed upon addition of sCD4-MPBH-fluorescein to CEM cells not exposed to HIV, addition of the conjugate to HIV-infected cells resulted in a significant shift in the mean fluorescence intensity from 3.8 to 5.6. It should be noted that this preparation of sCD4-MPBH-fluorescein contained some residual, unmodified sCD4. Indeed, a pure preparation of sCD4-MPBH-fluorescein would likely have been even more efficient in selectively labeling HIV-infected cells. Therefore, these results indicate that sCD4, conjugated via its carbohydrate using MPBH, may be used as a selective vector to target cells actively infected with HIV.

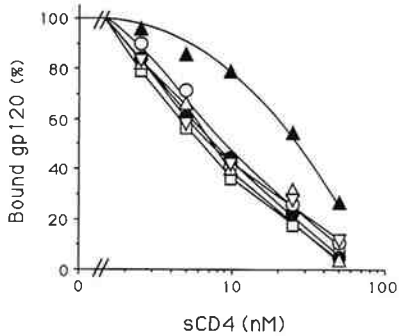

FIG. 5. **Binding of sCD4 conjugates to HIV-1 gp120.** sCD4 and its derivatives were tested for their ability to compete with immobilized CD4-IgG for the binding of ^{125}I-labeled gp120. sCD4 (□), sCD4-MPBH produced via the mild (●) or harsh (○) oxidation conditions, sCD4-MPBH-β-gal (△), sCD4-MPBH-fluorescein (▽), and sCD4-SMPB (▲). Unconjugated sCD4 was removed from sCD4-MPBH-β-gal by size exclusion chromatography (see "Materials and Methods").

DISCUSSION

In this paper we have described a novel cross-linking reagent, MPBH, which enables the coupling of glycoproteins, or other glycoconjugates, to thiol containing proteins or other compounds. Unlike cross-linking reagents such as SMPB, which are directed to free amino groups (for review, see Ref. 40), MPBH is carbohydrate-directed. Therefore, in the conjugation of glycoproteins such as sCD4, whose biological activity may be compromised by modification of amino groups, MPBH provides a useful alternative to conventional, amino group-directed cross-linkers.

Two important chemical properties are distinguishing features of MPBH: (i) its reactive moieties are a hydrazide and a maleimide, a stable nucleophile/electrophile pair (49, 50), and (ii) a 4-phenylbutanoyl spacer separates these functional groups, limiting the possibility of steric hindrance between the cross-linked compounds (41). The hydrazide and maleimide functional groups were chosen because they provide the desired reactivities with aldehydes and thiols. In addition, these groups are chemically compatible (particularly as the hydrazide hydrochloride), since they do not react with each other spontaneously, and thus self-polymerization of the reagent is prevented. In designing this cross-linker, we preferred a hydrazide over an amine functionality for reaction with aldehydes (28), since the former does not require that the conjugate be reduced. We considered combining a hydrazide function with another thiol-reactive moiety, a pyridyl disulfide (42, 43); however, the maleimide was preferred, since it forms a stable thioether linkage with thiols.

We demonstrated the ability of MPBH to conjugate a glycoprotein to a thiol-containing protein by producing stable conjugates of sCD4 with Hb and β-gal. Moreover, we showed that conditions could be tailored to achieve coupling of MPBH selectively through sialic acid residues of sCD4. The sCD4-β-gal conjugates were further characterized to determine their gp120 binding affinity relative to unmodified sCD4. The affinity was not changed significantly. Consistent with this finding, the gp120 binding affinity of sCD4 oxidized and coupled to MPBH, or of a sCD4-MPBH-fluorescein conjugate, was also unchanged. Furthermore, sCD4-MPBH-fluorescein was capable of labeling HIV-infected cells selectively, presumably by binding to HIV gp120 expressed on the surface of such cells. These results show that cross-linking of sCD4 to other molecules using MPBH has no adverse effect on its ability to bind to HIV gp120, thus confirming our prediction that conjugation of sCD4 via its carbohydrate moieties would not compromise its biological activity.

In contrast to the sCD4-MPBH conjugates, sCD4 coupled to the amino group-directed cross-linker SMPB exhibited a marked reduction in gp120 binding affinity. Consistent with

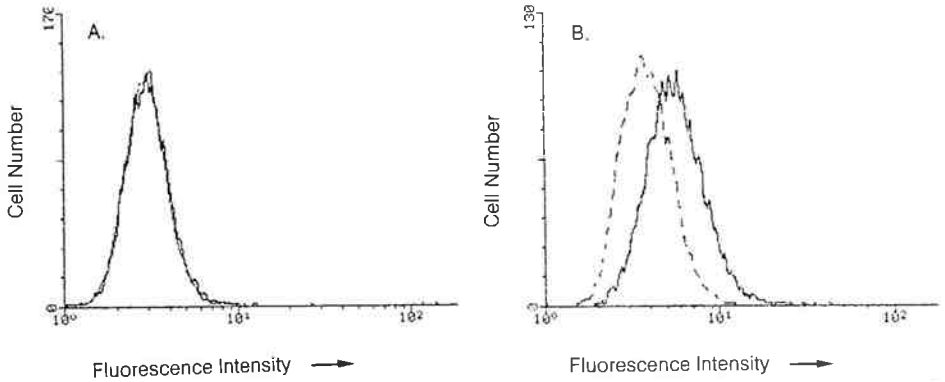

FIG. 6. **Selective staining of HIV-1-infected cells using sCD4-MPBH-fluorescein.** sCD4 was conjugated to fluorescein using MPBH, and the resulting sCD4-MPBH-fluorescein was tested for its ability to stain infected CEM.NKR cells in culture, as analyzed by FACS analysis. Uninfected cells (*A*) or HIV-1-infected cells (*B*) were incubated in the absence (*dashed line*) or presence (*solid line*) of sCD4-MPBH-fluorescein for 30 min on ice. The cells were washed, and green channel fluorescence was determined. For uninfected cells (*A*), the fluorescence intensity profiles, with or without sCD4-MPBH-fluorescein, were superimposable. For HIV-1-infected cells (*B*), the mean fluorescence intensity was 5.58 and 3.80 for cells incubated with or without sCD4-MPBH-fluorescein, respectively.

this result, Till *et al.* (20) reported a 50–75% reduction in gp120 binding affinity when sCD4 was conjugated to deglycosylated ricin A chain via an amino group-directed cross-linker. This differential effect of cross-linking strategies on affinity illustrates the unique advantage of a carbohydrate-directed cross-linker, such as MPBH, in conjugating to a glycoprotein in which certain amino groups are important for biological function. Thus, MPBH may facilitate conjugation of sCD4 to anti-HIV agents with no loss of gp120 binding affinity, and therefore, without adverse effects on targeting efficiency and selectivity. In addition to toxins as anti-HIV agents, liposomes containing toxins or agents which can inhibit HIV replication (44) may be targeted, via sCD4, to HIV-infected cells. To this end, MPBH has been used to produce sCD4-MPBH-liposomes which bind selectively to HIV-infected cells (45).

Indeed, use of MPBH should not be considered only when amino group-directed reagents may compromise the activity of the glycoprotein in question. For example, many cell surface receptors, including those for hormones, growth factors, and cytokines, are glycoproteins, and their carbohydrate moieties may be used to cross-link them to neighboring membrane components or to their ligands. Potential conjugation partners, in case they lack free thiols, can be thiolated by simple methods (46–48), once they are purified. In addition to glycoproteins, other glycoconjugates, for example glycophospholipids, should also be amenable to conjugation by this approach.

In conclusion, we have developed a novel cross-linking reagent, MPBH, which, unlike most available reagents, reacts with carbohydrates rather than with free amino groups. MPBH enables the conjugation of sCD4 to other compounds with no loss in its gp120 binding affinity, an effect which occurs with amino group-directed reagents. By its design, MPBH will be particularly useful for conjugating glycoproteins in which amino groups are important for function. In addition, this reagent may be useful for cross-linking any glycoprotein or sugar-containing compound to thiol-containing molecules.

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