Regulation of the Receptor Specificity and Function of the Chemokine RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) by Dipeptidyl Peptidase IV (CD26)-mediated Cleavage

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Summary

CD26 is a leukocyte activation marker that possesses dipeptidyl peptidase IV activity but whose natural substrates and immunological functions have not been clearly defined. Several chemokines, including RANTES (regulated on activation, normal T cell expressed and secreted), have now been shown to be substrates for recombinant soluble human CD26. The truncated RANTES(3–68) lacked the ability of native RANTES(1–68) to increase the cytosolic calcium concentration in human monocytes, but still induced this response in macrophages activated with macrophage colony-stimulating factor. Analysis of chemokine receptor messenger RNAs and patterns of desensitization of chemokine responses showed that the differential activity of the truncated molecule results from an altered receptor specificity. RANTES(3–68) showed a reduced activity, relative to that of RANTES(1–68), with cells expressing the recombinant CCR1 chemokine receptor, but retained the ability to stimulate CCR5 receptors and to inhibit the cytopathic effects of HIV-1. Our results indicate that CD26-mediated processing together with cell activation–induced changes in receptor expression provides an integrated mechanism for differential cell recruitment and for the regulation of target cell specificity of RANTES, and possibly other chemokines.

Monocytes differentiate into macrophages as they migrate from the blood to tissues during immune surveillance. At sites of inflammation, monocyte infiltration and macrophage accumulation are coordinated, in part, by chemokines (1). The mechanisms that control the recruitment of monocytes and macrophages by chemotactants have not been clearly defined, but they may include regulation of the expression of chemokines and their receptors (2) as well as the modification of chemokine activity by post-translational processing (3–5). Several chemokines share a conserved N-H2-X-Pro sequence (where X is any amino acid) at the NH2 terminus (6), which conforms to the substrate specificity of dipeptidyl exopeptidase IV (DPP IV; reference 7). DPP IV cleaves the first two amino acids from peptides with penultimate proline or alanine residues, although no natural substrate with immune function has been identified. This enzyme is also a leukocyte differentiation antigen, known as CD26 (8–10), that is expressed on the cell surface mostly by T lymphocytes and macrophages. Expression of CD26 has been associated with T cell activation (8–10) and with susceptibility of a T cell line to infection with macrophage-tropic HIV-1 (11).

In this study, we identify the chemokines RANTES (regulated on activation, normal T cell expressed and secreted), interferon-γ-inducible protein monocyte chemotactic protein (MCP)-2, eotaxin, and IP-10 as the first natural CD26 substrates with immune function. It is shown that the cleavage product of RANTES is a chemokine agonist with al-

Abbreviations used in this paper: [Ca2+]i, cytosolic free Ca2+ concentration; DPP IV, dipeptidyl peptidase IV; E1, enzymatically active; E2, enzymatically deficient; ES-MS, electrospray mass spectrometry; GAPDH, glycer-aldehyde phosphate dehydrogenase; HEK, human embryonic kidney; HOS, human osteosarcoma; IP, interferon-γ-inducible protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; pNA, p-nitroanilide; RANTES, regulated on activation, normal T cell expressed and secreted; rh, recombinant human; s, soluble; SDF, stromal-derived factor.
tered receptor specificity. We also describe, for the first time, differential changes in the expression pattern of chemokine receptors after activation of monocytes by M-CSF. Therefore, target cell recruitment into inflammatory sites may depend both on the extent of CD26 activity on chemokines and on the maturational status of the responding cells.

Materials and Methods

Cell Cultures and Transfections. Monocytes were isolated from human PBMCs of healthy donors by counter-current centrifugal elutriation. Monocyte-derived macrophages were prepared by culturing monocytes for 6 d at a density of 10^6 cells/ml in serum-free macrophage medium (GIBCO BR L, Gaithersburg, MD) supplemented with recombinant human (rh) M-CSF (10 ng/ml; R&D Systems, Inc., Minneapolis, MN).

Human embryonic kidney (HEK)-293 cells grown to confluence in DMEM supplemented with 10% heat-inactivated FCS, penicillin, streptomycin, 2 mM glutamine, and 10 mM Hepes (pH 7.4) were transfected with plasmid DNA encoding CRCR5 (12). CD4-positive human osteosarcoma (HOS-CD4) cell lines transfected with individual chemokine receptor cDNAs were obtained from N. Landau (Aaron Diamond AIDS Research Center, New York), and were grown in the above culture medium supplemented with 0.1% (vol/vol) glacial acetic acid, using a Finnigan mass spectrometry trap (Michrom BioResources, Inc., Auburn, CA), or a reversed-phase-HPLC detector. Monocytes were isolated from human PBMCs of healthy donors by counter-current centrifugal elutriation. Monocyte-derived macrophages were prepared by culturing monocytes for 6 d at a density of 10^6 cells/ml in serum-free macrophage medium (GIBCO BR L, Gaithersburg, MD) supplemented with recombinant human (rh) M-CSF (10 ng/ml; R&D Systems, Inc., Minneapolis, MN).

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...that had been premixed with 10% Pluronic F-127 (Molecular Probes). The cells were then washed and resuspended at 2 × 10^6 cells/ml in Ca^2+ buffer containing BSA (1 mg/ml), and portions (2 ml) of the cell suspension were exposed at different time points in a stirred cuvette at 37°C to chemokines. Fluorescence was monitored with a 2 scan (Photon Technology Intl., Monmouth Junction, NJ), and data were recorded at the relative ratio of fluorescence at excitation wavelengths of 340 and 380 nm, with emission measured at 510 nm. After each measurement, maximal and minimal fluorescence were assessed by addition of 20 μM ionomycin followed by 5 mM MgCl2.

A assay for HIV-1-induced Cytotoxicity. HO S-C D4 CCR5 cells (2 × 10^6) were incubated for 1 h at 37°C with RANTES variants in 150 μl of culture medium containing 20% FCS, and were then mixed with 50 μl (2 × 10^6 cells/ml) of uninfected PM 1 cells or PM 1 cells chronically infected with MV3-H X B2 virus. After 3 d, photomicrographs of cultures were taken and cell viability was measured by adding of 50 μl of 1 mg/ml 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide solution containing 20 μM phenazine methosulfate and recording the OD at 450 nm. Data are expressed as the percentage of inhibition of cytopathicity (calculated as 100% × (R - V)/(U - V)), where U, V, and R represent OD values obtained for HOS-C D4 CCR5 cells cultured with uninfected PM 1 cells or with HIV-1-infected cells in the absence or presence of chemokine, respectively.

Results

RANTES, MCP-2, eotaxin, and IP-10 Are Substrates of CD26. ES-MS analysis revealed that 100 nM rhRANTES underwent partial to complete hydrolysis when incubated overnight at 37°C with increasing amounts (25–250 μg) of scCD26 (Fig. 1). Taking into account cationization (K^+) of the multiply charged ions, the measured molecular masses of the native and degraded polypeptides corresponded to the theoretical masses of full-length (residues 1–68) and rhRANTES(1–68) as judged by the Ca^2+ influx and anti-HIV-1 assays used in this study.

Colorimetric DPP IV Enzyme Assay. The p-nitroanilide (pNA)-conjugated Gly-Pro dipeptide substrate and test competitors were mixed and added to human placental DPP IV (Enzyme Systems Products, Dublin, CA), and the resulting mixture was incubated at room temperature in a final volume of 150 μl containing 50 mM tris-HCl (pH 8.0) and 0.15 M NaCl. The final concentrations of DPP IV and Gly-Pro-pNA were 1.25 μM/ml and 400 μM, respectively. The kinetics of the enzyme reaction were monitored by measuring absorbance at 405 nm with a Max kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA). The percentage of inhibition of enzyme activity was calculated from the maximal velocity for each sample and from that apparent in the absence of competitor (100% activity).
and truncated (residues 3–68) forms of RANTES, respectively. The calculated difference between the molecular masses of the native and the truncated forms ranged from 183 to 185 daltons, which is consistent with the expected mass (184 daltons) of a released Ser-Pro dipeptide, the predicted NH$_2$-terminus of RANTES (16). In contrast to the effect of enzymatically active sCD26, shortened RANTES was not generated by incubation of the chemokine with a mutant sCD26 deficient in enzyme activity (Fig. 1). RANTES also inhibited, possibly in a competitive manner, the rapid hydrolysis of a pNA-conjugated Gly-Pro dipeptide by human placental DPPIV, as measured in a colorimetric enzyme assay (Fig. 2). The efficacy of inhibition by chemically synthesized RANTES(1–68) was similar to that observed with the DPPIV substrate and competitive inhibitor Ile-Pro-Ile (Diprotin A; reference 17), whereas RANTES(3–68) did not inhibit the reaction.

Sensitivity to CD26-mediated cleavage was not a unique property of RANTES (Table 1). Cleavage products with the predicted molecular masses were also evident in samples of MCP-2, eotaxin, and IP-10 after incubation with sCD26. In contrast, MCP-1, which has a 62% sequence similarity with MCP-2 including the NH$_2$-terminal Gln-Pro dipeptides, was not cleaved by the enzyme under the same experimental conditions.

CD26-specific truncation of RANTES modifies its target cell specificity. To investigate the functional significance of DPPIV-mediated truncation of RANTES, we compared the effects of chemically synthesized RANTES(1–68) and RANTES(3–68) with the predicted molecular masses were also evident in samples of MCP-2, eotaxin, and IP-10 after incubation with sCD26. In contrast, MCP-1, which has a 62% sequence similarity with MCP-2 including the NH$_2$-terminal Gln-Pro dipeptides, was not cleaved by the enzyme under the same experimental conditions.

**Table 1.** Chemokine cleavage products after digestion with sCD26

| Chemokine | NH$_2$-terminal dipeptide | CD26 cleavage | Molecular masses by mass spectrometry (Da) |
|-----------|---------------------------|---------------|------------------------------------------|
|           |                           |               | Full length | Truncated |
|           |                           | Theoretical   | Observed  | Theoretical | Observed  |
| Eotaxin   | GP                        | Yes           | 8,361     | 8,361       | 8,207     | 8,207     |
| IP-10     | VP                        | Yes           | 8,633     | 8,637/8,751*| 8,437     | 8,440/8,555*|
| MCP-1     | QP                        | No            | 8,681     | 8,678       | 8,456     | N D$^\dagger$|
| MCP-2     | QP                        | Yes           | 8,910     | 8,909       | 8,685     | 8,686/8,703$^\ddagger$|

$^*$ Tentatively identified as [M + trifluoroacetic acid (TFA)]$^+$; molecular mass of TFA is 114 Daltons.
$\dagger$ Tentatively identified as [M + H2O]$^+$. 
$^\ddagger$ ND, not detected.
and RANTES(3–68) on monocytes and monocyte-derived macrophages. Both resting cells and cells activated with M-CSF were analyzed because reverse transcriptase–PCR revealed marked changes in the abundance of chemokine receptor transcripts in response to M-CSF activation (Fig. 3). In resting cells, transcripts encoding the chemokine receptors CCR1, CCR2b, or CXCR4, as well as control GAPDH mRNA, were readily detectable, whereas CCR5 receptor transcripts were virtually absent. After differentiation to macrophages, the intensity of the CXCR4 and GAPDH signals remained virtually unchanged, whereas the abundance of CCR1 and CCR5 mRNA increased substantially and the CCR2b transcript virtually disappeared. CCR3 mRNA was not detected in either cell type.

Transient changes in the cytosolic free Ca²⁺ concentration ([Ca²⁺]) were recorded after stimulation of monocytes or macrophages with an optimal concentration of RANTES (1–68) or RANTES(3–68), and the effects were compared with those of other chemokines (Fig. 4). Addition of 100 nM RANTES(1–68) to cells loaded with the fluorescent Ca²⁺ probe Fura-2 induced a rapid increase in [Ca²⁺]i in both monocytes and macrophages. In contrast, the same concentration of RANTES(3–68) increased [Ca²⁺]i in macrophages but not in monocytes. Among the other chemokines tested, macrophage inflammatory protein (MIP)-1α, MCP-1, MCP-3 (1, 6), and stromal-derived factor-1β (SDF-1β; references 18–20) also increased [Ca²⁺]i, in resting monocytes, whereas MCP-2 (21) induced a barely detectable response and MIP-1β (1, 6) was inactive. On the basis of the previously described receptor specificities of these chemokines (1, 6, 19, 20), the obtained activity pattern is consistent with expression of CCR1, CCR2b, and CXCR4 receptors on monocytes (Fig. 3). Macrophages showed marked Ca²⁺ responses to MIP-1α, MIP-1β, MCP-1, MCP-3, and SDF-1β, but were resistant to MCP-2, consistent with the presence of transcripts encoding CCR1, CCR5, and CXCR4, and the absence of those encoding CCR2b in these cells (Fig. 3).

RANTES(3–68) is a C chemokine Agonist, with A itered Receptor Specificity. Agonists that act at common chemokine receptors block each other’s activity as a result of receptor desensitization, whereas responses to chemokines that act at different receptors generally are not affected (1, 6). Therefore, we performed comparative desensitization experiments to define the types of receptors that mediate the effects of native versus truncated RANTES in macrophages (Fig. 5). Macrophages that were stimulated first with 100 nM RANTES(1–68) did not exhibit a second Ca²⁺ response when challenged with the same dose of either full-length or truncated RANTES. In contrast, cells stimulated with 100 nM RANTES(3–68) fully retained their ability to respond to a subsequent challenge with full-length RANTES, but were desensitized to the effect of the truncated form. These results suggest that the receptor repertoire available for truncated RANTES is more restricted than that available for the native chemokine. To further characterize the receptor usage of the different forms of RANTES and other chemokines, we also studied the sensitivity of MIP-1β-, MCP-3-, and SDF-1β–induced Ca²⁺ responses to RANTES-mediated receptor desensitization (Fig. 5). Of the known receptors, RANTES signals via CCR1, CCR4, and CCR5, whereas MIP-1β acts at CCR5 exclusively and MCP-3 binds only to CCR1 and CCR2b at the concentrations used in our experiments (1, 6). The only receptor known to bind SDF-1β is CXCR4 (19, 20). Pretreatment of macrophages with full-length RANTES blocked the ability of MIP-1β and MCP-3, but not that of SDF-1β, to increase [Ca²⁺]. In contrast, RANTES(3–68) desensitized cells to the effect of MIP-1β but did not affect the response to MCP-3 or SDF-1β. These results are consistent with previous data on RANTES-induced receptor desensitization (1) and with our data on chemokine receptor mRNA abundance (Fig. 3). They suggest that, in M-CSF–activated macrophages, full-length RANTES shares CCR1 and CCR5 receptors with MCP-3 and MIP-1β, respectively. Our results also indicate that, without its two NH₂-terminal residues, RANTES is still able to signal via CCR5 but can no
longer act at the CCR1 receptor.

Removal of the NH₂-Pro residues affects the CCR1-, but not the CCR5-mediated signaling of RANTES. HEK-293 cells expressing CCR5 and HOS-CD4 cells expressing CCR1 were loaded with Fura-2 and exposed to various concentrations of RANTES(1–68) or RANTES(3–68). The two RANTES variants showed similar abilities to increase [Ca²⁺], in the CCR5 transfectant (Fig. 6 A); the responses were dose dependent, with 10 nM of each variant sufficient to induce a maximal Ca²⁺ response. In contrast, in the cells expressing CCR1, the amount of RANTES(3–68) required to produce a detectable Ca²⁺ response was ~100 times that for RANTES(1–68) (Fig. 6 B); the effect of RANTES(1–68) saturated at 50 nM, whereas that of RANTES(3–68) appeared not to have achieved saturation at 200 nM. Furthermore, bidirectional cross-desensitization between the two RANTES variants was evident only with the cells expressing CCR5 (Fig. 6 C); in the CCR1 transfectant, cross-desensitization was induced by full-length RANTES but not by the truncated form, which also did not exhibit self-desensitization (Fig. 6 D). Control cells transfected with vector alone or with vectors encoding CCR2b, CCR3, or CXCR4 did not respond to these ligands (data not shown). These results thus confirm that the native and CD26-truncated RANTES variants exhibit markedly different activities at the CCR1 receptor.

RANTES(3–68) is a potent inhibitor of HIV-1. In addition to their function in chemotaxis, RANTES, MIP-1α,
and MIP-1β each inhibit HIV-1 infection by competitive binding to CCR5 (22–27), and this inhibition does not require receptor-mediated cell signaling (27, 28). To examine whether removal of the two NH₂-terminal residues affects the antiviral activity of RANTES, we mixed HOS-CD4 cells expressing recombinant CCR5 and PM1 cells chronically infected with the macrophage-tropic recombinant MV3-HXB2 virus and cocultured them in the absence or presence of various concentrations of RANTES(1–68) or RANTES(3–68). Both RANTES variants inhibited HIV-1-induced syncytium formation and cytopathicity (Fig. 7). Thus, similar to signaling activity through CCR5, competitive inhibition of HIV-1 infection does not require the NH₂-terminal Ser-Pro residues of RANTES.

Discussion

Chemical modifications at the NH₂ terminus of chemokines have been previously suggested to produce polypeptides that are antagonists of the native chemoattractants (3, 4). However, these alterations did not correspond to the physiological specificities of known enzymes and no natural equivalents of the modified chemokines were identified. In contrast, the CD26 cleavage product of RANTES, RANTES(3–68), acts as a chemokine agonist with altered receptor specificity. Hydrolysis by CD26 might explain why RANTES(3–68) has been isolated as a second component in addition to intact RANTES from culture supernatants of stimulated human fibroblasts, skin samples, and platelet preparations (29, 30). The CC-chemokines RANTES, MCP-2, and eotaxin, and the CXC-chemokine IP-10 are the first immune modulators and the longest polypeptides identified as natural substrates for CD26.

CD26 exists in both soluble and membrane-expressed forms. Secreted forms of CD26 have been identified in cell cultures and in human serum (31, 32), although CD26 may be more active when expressed as an ectoenzyme at high concentrations on endothelial cells, hepatocytes, kidney brush border membranes, and leukocytes (10). Upregulation of CD26 expression on T lymphocytes and macrophages has been linked to cell activation and development of immunological memory (10). Thus, activation-induced changes in CD26 expression could affect the course of an inflammatory response by modifying the target cell specificity of RANTES or other chemokines, and by regulating the equilibrium between the migrating cell subsets. We are currently addressing whether cells with different levels of CD26 expression (e.g., naïve versus memory T cells) secrete truncated forms of RANTES or other chemoattractants, or are capable of modifying exogenous chemokines.

The differential effects of CD26-truncated RANTES on monocytes versus macrophages illustrate a role for cell differentiation in regulating chemokine sensitivity through altered receptor expression. Our functional and receptor transcript data indicate that CCR1 and CCR2b may be the two principal CC chemokine receptors in resting monocytes, although other unidentified and functionally overlapping receptors may also contribute to chemokine function. Cell differentiation markedly changes the pattern of chemokine sensitivity by reducing CCR2b expression, thereby rendering the cells resistant to MCP-1, while increasing CCR5 expression, thereby augmenting the responses to CD26-truncated RANTES and MIP-1β. An increase in CCR5 expression may also render macrophages more susceptible to infection by macrophage-tropic variants of HIV-1. We have shown that macrophages also express CXCR4, the coreceptor for T cell line-tropic HIV-1 variants (33, 34), as assessed by receptor transcript abundance and functional activity of the CXCR4 ligand SDF-1β. Nevertheless, activated macrophages are relatively re-
sistant to infection by T cell line-tropic HIV-1 variants (35), which suggests that factors other than CXCR 4 may also be required for efficient infection of macrophages by these types of viruses.

Removal of two NH2-terminal residues by CD26 abolishes the interaction of RANTES with CCR 1, but does not affect the anti-HIV-1 activity or the CCR 5 signaling properties of the chemokine. Proline residues also influence the susceptibility of proximal peptide bonds to proteolytic enzymes (6), and so the removal of such residues by CD26 may also reduce the half-life of RANTES and other chemokines during an inflammatory response. It will be important to determine whether CD26-mediated cleavage is a general mechanism for changing the receptor specificity and functional activity of other chemokines, including those examined in this study (MCP-2, eotaxin, and IP-10).

We thank K. Faust and V. Calvert for help in separation of mononuclear cells, C. Abbott for mutant CD26 cDNAs, and M. Samson for the CCR 5 vector. The HOS-C4D cell lines were obtained from N. Landau through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases).

This study was supported in part by funds from the National Institutes of Health AIDS Targeted Antiviral Program and from the National Health and Medical Research Council of Australia (M.D. Gorrell).

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Received for publication 4 June 1997 and in revised form 22 September 1997.

Note added in proof. We note that a CD8+ T cell–derived HIV-1 suppressor activity has been recently identified as a truncated form of macrophage-derived chemokine (MDC), missing a glycine–proline dipeptide from the NH2 terminus (Pal, R., A. Garzino-Demo, P.D. Markham, J. Burns, M. Brown, R.C. Gallo, and A.L. DeVico. 1997. Science. 278:695–698). Based on our results, we suggest that truncation of MDC is a consequence of CD26-mediated cleavage that may have resulted in enhanced MDC antiviral activity.

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