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N36, a Synthetic N-Terminal Heptad Repeat Domain of the HIV-1 Envelope Protein gp41, Is an Activator of Human Phagocytes[1]

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Human immunodeficiency virus type 1 (HIV-1) envelope protein gp41 mediates viral fusion with human host cells. In this study we show that N36, a synthetic peptide derived from the N-terminus of gp41, induced directional migration and calcium mobilization in human monocytes and neutrophils. The activity of N36 on phagocytes was pertussis toxin sensitive, suggesting involvement of a Gi-coupled seven-transmembrane receptor(s). Since high concentrations of the bacterial chemotactic peptide fMet-Leu-Phe (fMLF) partially desensitized the calcium mobilizing activity of N36 in phagocytes, we postulated that N36 might use a low-affinity fMLF receptor. By using cells stably expressing fMLF receptor FPR or FPRL1, we demonstrate that N36 uses FPRL1 as a functional receptor. Our results suggest that HIV-1 gp41 may contain a fragment(s) that activates the innate host immune cells through FPRL1. Since the activation of FPRL1 in monocytes has been shown to heterologously desensitize chemokine receptors, the reduced phagocyte response to chemoattractants seen in AIDS patients may be attributed, at least in part, to heterologous desensitization.

Key Words: **N36 peptide; HIV-1 gp 41; chemoattractant receptor; phagocyte.**

INTRODUCTION

Human immunodeficiency virus (HIV) uses envelope glycoproteins to enter cells. The envelope glycoprotein complex consists of the surface subunit gp120 and the transmembrane subunit gp41, which are produced through proteolytic cleavage of the precursor gp160. gp120 binds to CD4 and one of several coreceptors that are members of the chemokine receptor family. Subsequently, gp41 undergoes conformational changes that facilitate fusion of viral and host cell membranes (reviewed in Refs. 1, 2). The ectodomain of gp41 contains a glycine-rich N-terminal sequence, named the fusion peptide, that is essential for membrane fusion (Fig. 1). The fusion peptide region is followed by two 4–3 hydrophobic (heptad) repeat regions predicted to form coiled-coils. The N-terminal heptad repeat region is located adjacent to the fusion peptide, while the C-terminal heptad repeat region precedes the transmembrane segment (Fig. 1). Synthetic peptides derived from the N- and C-terminal heptad repeat regions, designated the N and C peptides, respectively, are effective inhibitors of HIV infection and syncytia formation (3–5). The anti-HIV type 1 (HIV-1) activity of these peptides is proposed to be due to their competitive association with the corresponding segments on gp41, thus blocking the fusion between the viral and the cellular membranes.

Limited proteolysis of a recombinant fragment corresponding to the gp41 ectodomain generated a trimeric, α-helical complex composed of two peptides, designated N51 and C43, that are derived from the N- and C-terminal heptad repeat regions (6). By further protein dissection, a subdomain within gp41 composed of the N36 and C34 peptides was identified (7). This complex is believed to represent the core of fusion-active gp41 (8–10).

We have investigated the mechanistic basis for HIV-1-associated suppression of monocyte function and found that preexposure of human monocytes to either HIV-1 envelope proteins gp120 or gp41 inhibited their chemotactic response to a wide variety of chemoattractants, including the bacterial chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) and a number of chemokines, through a mechanism resembling heterologous "desensitization" (11, 12). The inac-

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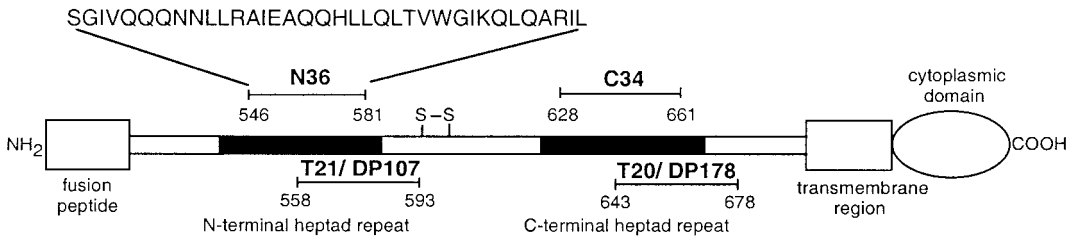

FIG. 1. Schematic representation of gp41. N and C peptides derived from the N- and C-terminal heptad repeat regions are indicated. The residues are numbered according to their positions in gp160 (Ref. 8).

tivation of monocytic chemotactic responses by HIV-1 envelope proteins may be responsible for the reduced migratory response of monocytes from AIDS patients to various chemoattractants *in vitro* (13). In order to define the structural basis for the capacity of HIV-1 envelope proteins to "desensitize" host cells, we evaluated the effects of selected peptide segments of gp41 on human immune cells. We initially found that a synthetic C peptide, T20/DP178 (Fig. 1), was a potent chemoattractant and activator of human phagocytic leukocytes. The effect of T20/DP178 was mediated by FPR, a seven-transmembrane, G-protein-coupled receptor originally identified as the receptor for bacterial chemotactic formyl peptides (14–16). On the other hand, the synthetic N-terminal domain of gp41, T21/DP107 (Fig. 1), activated human phagocytes by using both FPR and a FPR variant, FPRL1, as its functional receptors (17). Since N36 and C34 regions have been implicated as key structures for conversion of gp41 to the fusogenic conformation (8–10) and they overlap with T21/DP107 and T20/DP178 segments (Fig. 1, and Refs. 5–7), we determined whether these peptide domains had any potential to interact with human immune cells. Here we report that N36 induces the directional migration and activation of human phagocytic cells by preferentially stimulating the receptor FPRL1.

MATERIALS AND METHODS

Reagents and Cells

N36 peptide, SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL, which corresponds to amino acid residues 546–581 in the N-terminal heptad repeat region of HIV-1 gp41, was synthesized by a standard solid-phase FMOC method in the New York Blood Center in-house facility. The N-terminus of the peptide was uncapped and its C-terminus was amidated. The peptide was purified to homogeneity by high-performance liquid chromatography. The identity of the purified peptides was confirmed by laser desorption mass spectrometery (PerSeptive Biosystems). The endotoxin levels in dissolved peptide were undetectable by *Limulus amebocyte* lysate assays (sensitivity 0.06 IU/ml; Bio-Whittaker). Several other peptides from HIV-1 gp41, including fusion peptide (FP, aa 512–527), C34 (aa 628–661, a kind gift from Dr. P. S. Kim, Massachusetts Institute of Technology, Cambridge, MA), and recombinant N36(L6)C34, a stable subdomain consisting of N36 and C34, connected by a six-residue hydrophilic linker (a kind gift from Dr. M. Lu, Cornell University Medical College, New York, NY), were also tested for their biological effects. The synthetic chemotactic peptide fMLF was purchased from Sigma (St. Louis, MO). Recombinant human serum amyloid A (rhSAA) and chemokines were purchased from PeproTech Inc. (Rocky Hill, NJ). Human peripheral blood monocytes were isolated from Buffy Coats (NIH Clinical Center, Transfusion Medicine Department, Bethesda, MD) enriched for mononuclear cells by using an iso-osmotic Percoll gradient. Neutrophils were isolated from Buffy Coat blood with dextran sedimentation. The purity of the cell preparations was examined by morphology and was >90% for monocytes and >98% for neutrophils. Rat basophilic leukemia cells (RBL-2H3) transfected with epitope-tagged FPR (designated ETFR) were a kind gift from Drs. H. Ali and R. Snyderman (Duke University, Durham, NC). FPRL1-transfected HEK/293 cells (designated FPRL1/293) were a kind gift from Drs. Philip M. Murphy and Jiliang Gao (NIH, Bethesda, MD). All the transfected cells were maintained in DMEM supplemented with 10% FBS (Hyclone, Logan, UT), 1 mM glutamine (Gibco BRL, Grand Island, NY), and 800 μg/ml geneticin (G418, Gibco BRL).

Chemotaxis and Calcium Mobilization Assays

Chemotaxis assays were performed using 48-well chemotaxis chambers (Neuro Probe, Cabin John, MD) as described previously (14, 17). The results were expressed as the chemotaxis index, which represents the fold increase in the number of migrating cells in three high-powered fields in response to chemoattractants over the spontaneous cell migration in response to control medium. Ca^{2+} mobilization was measured by incubating 2×10^7 cells/ml in loading medium

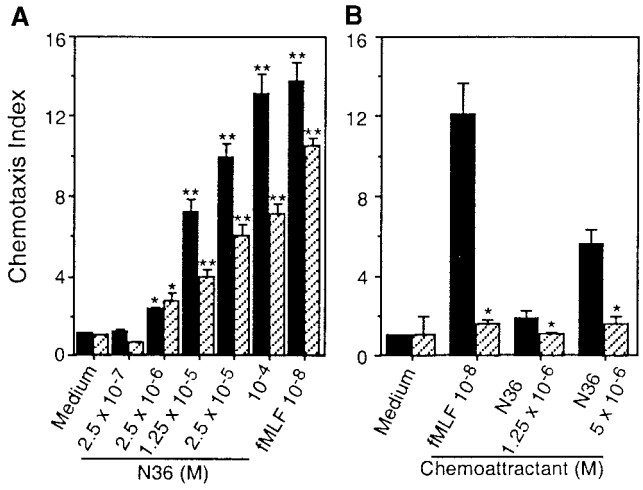

FIG. 2. Chemotactic activity of N36 peptide for monocytes and neutrophils. (A) Fold increase (chemotaxis index) of phagocyte migration in response to N36 peptide over control medium (black histogram, monocytes; hatched histogram, neutrophils). $*P < 0.05$, $**P < 0.01$, compared with spontaneous migration. (B) Inhibition of monocyte migration in response to N36 peptide by pertussis toxin (black histogram, cells were incubated with medium; hatched histogram, cells were preincubated with 100 ng/ml pertussis toxin at 37°C for 30 min, washed, and examined for migration induced by different concentrations of N36 peptide). $*P < 0.01$ compared with migration of cells incubated with medium alone. fMLF was used as a control.

(DMEM, 10% FBS, 2 mM glutamine) with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR) for 30 min at room temperature. The dye-loaded cells were washed and resuspended in saline buffer (138 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 10 mM Hepes (pH 7.4), 5 mM glucose, 0.1% BSA) or HBSS at a density of 1×10^6/ml. The cells were then transferred into quartz cuvettes ($1–2 \times 10^6$ cells in 2 ml) that were placed in a luminescence spectrometer (LS-50B, Perkin–Elmer, Beaconsfield, England). Stimulants at different concentrations were added in a volume of 20 μl to each cuvette at the indicated time points. The ratio of fluorescence at 340 and 380 nm was calculated using a FL WinLab program (Perkin–Elmer).

Statistical Analysis

All experiments were performed at least three times and the results presented are from representative experiments. The significance of the difference between test and control groups was analyzed with Student's *t* test.

RESULTS

We first tested whether synthetic N36, FP, and C34 and the recombinant N36(L6)C34 could induce human leukocyte migration, a crucial step for cell homing and accumulation at sites of inflammation or injury. While FP, C34, and N36(L6)C34 did not induce significant migration and calcium mobilization in human peripheral blood monocytes and neutrophils (data not shown), both these cell types migrated in a dose-dependent manner in response to a concentration gradient of N36 (Fig. 2A). Checkerboard analyses showed that monocytes migrated when higher concentrations of N36 were present in the lower wells of the chemotaxis chamber (Table 1). Cell migration was not enhanced when higher concentrations of N36 were present in the upper wells or equal concentrations of N36 were in both upper and lower wells. These results suggest that the effect of N36 on monocyte migration was chemotactic rather than chemokinetic. The migration of monocytes to N36 was completely inhibited by pretreatment of the cells with pertussis toxin (Fig. 2B), suggesting that a receptor coupled to a Gi-type G protein (15, 16, 18) was involved in cell activation by N36. This hypothesis was supported by the induction of a dose-dependent and pertussis toxin-sensitive Ca^{2+} mobilization in monocytes and neutrophils with N36 (Figs. 3A and 3C; and data not shown).

To define the receptor(s) possibly used by N36 on phagocytic cells, a series of cross-desensitization experiments was performed by using a variety of chemoattractants. N36 did not desensitize the Ca^{2+} flux in monocytes or neutrophils induced by chemokines such as monocyte chemoattractant protein-1, regulated on activation normal T-cell expressed and secreted, monocyte chemoattractant protein-3, macrophage inflammatory protein-1 α, interleukin 8, and stromal cell-

TABLE 1

Checkerboard Analysis of Monocyte Migration in Response to N36 Peptide[a]

N36 peptide in lower wells (M)	Number of migrated cells in 1 HPF (mean ± SE)			
	N36 peptide in upper wells (M)			
	Medium	1.25×10^{-6}	5.00×10^{-6}	1.25×10^{-5}
Medium	8 ± 2	7 ± 1	3 ± 1	3 ± 1
1.25×10^{-6}	18 ± 2[b]	5 ± 1	2 ± 1	2 ± 1
5.00×10^{-6}	56 ± 10[b]	28 ± 6[b]	6 ± 1	5 ± 1
1.25×10^{-5}	78 ± 8[b]	36 ± 6[b]	25 ± 4[b]	10 ± 2

[a] Different concentrations of N36 peptide were placed in the upper and/or lower wells of the chemotaxis chamber; monocytes at 2×10^6/ml were placed in the upper wells. The upper and lower wells were separated by a polycarbonate filter. After incubation, the nonmigrating cells were removed, the filter was fixed and stained, and the cells that migrated across the filter were counted in three high-powered fields (HPF, 400×). The results are expressed as the mean value (±SE) of the cells in 1 HPF. Similar results were obtained for neutrophils in two separate experiments.

[b] $P < 0.01$ compared with migration in the presence of medium alone in both upper and lower wells as determined by Student's *t* test.

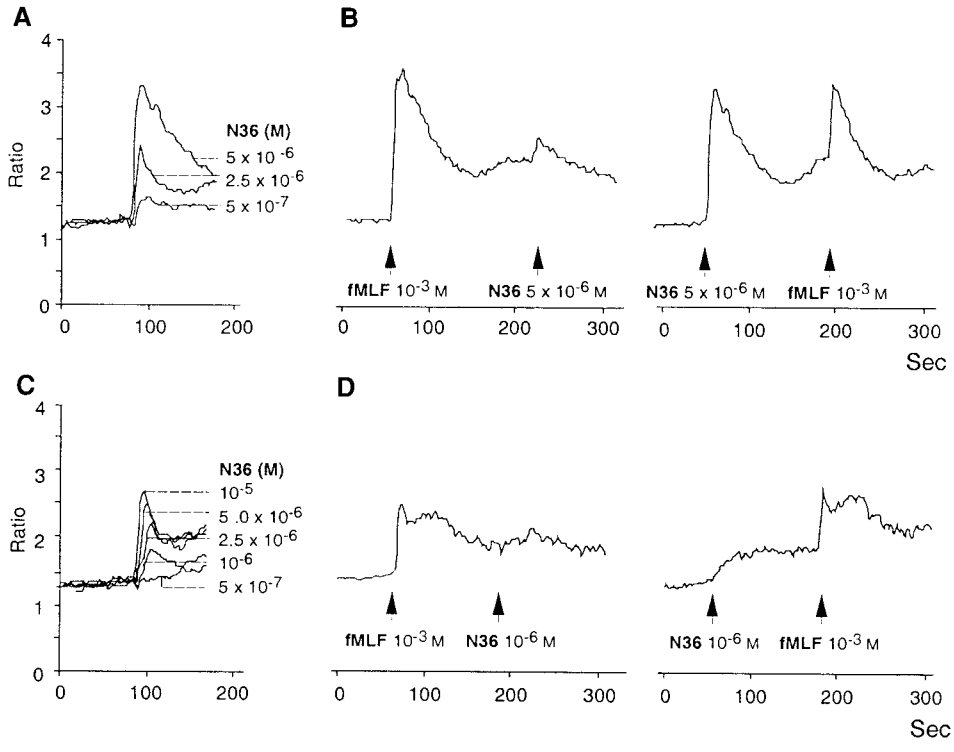

FIG. 3. Calcium mobilization induced by N36 peptide in phagocytes and attenuation by fMLF. Cells were loaded with Fura-2 and stimulated with different concentrations of N36 peptide or sequentially stimulated with fMLF and N36 peptide or vice versa (A and B, neutrophils; C and D, monocytes).

derived factor-1 α (data not shown). Therefore, N36 does not share a receptor with any of these chemokines. However, a high concentration (10^{-3} M) of the bacterial chemotactic N-formylated peptide fMLF had a significant desensitizing effect on N36-induced Ca^{2+} mobilization in both monocytes and neutrophils (Figs. 3B and 3D). In contrast, N36 only weakly desensitized the effect of fMLF (Figs. 3B and 3D). These results suggest that on human phagocytic cells N36 and fMLF may share a receptor that has low affinity for fMLF.

Two G protein-coupled seven-transmembrane receptors that can be activated by fMLF have been identified: the high-affinity receptor FPR and its variant FPRL1 (15, 16). We tested the signaling of N36 peptide on cells transfected with cDNAs coding either FPR (designated ETFR cell) or FPRL1 (designated FPRL1/293 cell). FPRL1/293 cells, but not ETFR cells or mock-transfected 293 cells, were responsive to N36 peptide in both chemotaxis (Fig. 4A; and data not shown) and Ca^{2+} mobilization experiments (Figs. 5A and 5B; and data not shown). As observed in monocytes, the migration of FPRL1/293 cells induced by N36 was also inhibited by pretreatment of the cells with pertussis toxin (Fig. 4B). In addition, while the Ca^{2+} mobilization induced by N36 peptide in FPRL1/293 cells was attenuated by high concentrations of fMLF, N36 peptide conversely attenuated fMLF-induced Ca^{2+} mobilization in these cells (Fig. 5C). The concentrations of N36 peptide required to activate FPRL1/293 cells were in the low micromolar range, comparable to those for phagocyte activation. These results indicate that N36 peptide uses FPRL1 as a functional receptor. We recently identified FPRL1 to be a functional receptor for a normal human serum protein, serum amyloid A (SAA) (19), which increases its concentration by up to several hundredfold during acute-phase responses and is a potent phagocyte chemoattractant and activator (20, 21). Therefore we tested the cross-desensitization of Ca^{2+} flux in monocytes and FPRL1/293 cells between N36 and SAA to further confirm the usage of FPRL1 by N36. SAA and N36 attenuated each other's Ca^{2+} mobilizing activity in both monocytes and FPRL1/293 cells (Figs. 5D and 5E), indicating that these two chemoattractants share FPRL1 as their functional receptor. It should be noted that N36 does not bear any significant sequence homology to either fMLF or SAA. Therefore, FPRL1 is capable of reacting with a broad spectrum of ligands, a characteristic feature also reported for the prototype receptor FPR (15, 16).

DISCUSSION

FPRL1 was identified and molecularly cloned a number of years ago, it possesses 69% identity to FPR at the

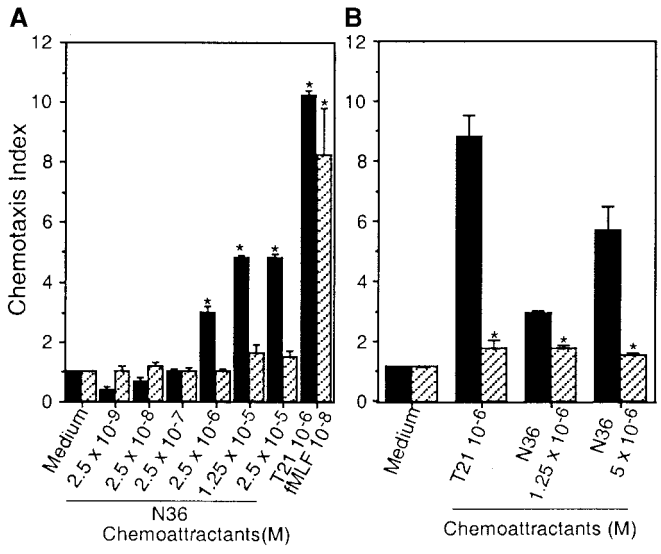

FIG. 4. Chemotactic activity of N36 peptide for FPRL1/293 cells. (A) Fold increase (chemotaxis index) of FRPL1/293 cell migration (black histogram) or ETFR cell migration (hatched histogram) in response to N36 peptide over control medium. T21/DP107 and fMLF were used as control. (B) Inhibition of FPRL1/293 cell migration in response to N36 peptide by pertussis toxin (black histogram, cells were incubated with medium; hatched histogram, cells were preincubated with 100 ng/ml pertussis toxin at 37°C for 30 min, washed, and examined for migration induced by different concentrations of N36 peptide). $*P < 0.01$ compared with migration of cells incubated with medium alone. T21 was used as control.

amino acid level (15, 16). Both receptors are expressed by monocytes and neutrophils and are clustered on human chromosome 19q13. While fMLF is a high-affinity agonist for FPR, it interacts with and induces Ca^{2+} flux in FPRL1 only at high concentrations. Furthermore, fMLF did not induce significant migration of FPRL1/293 cells at a concentration as high as 50 μM (17, 19), suggesting that fMLF is not a full agonist for FPRL1. In contrast, N36 induces migration of FPRL1/293 cells at low micromolar concentrations. Thus, compared to fMLF, N36 is a functionally more relevant agonist for FPRL1. Although the signal transduction pathways mediated by FPRL1 have not been extensively studied, the high level of homology to FPR, sensitivity to pertussis toxin, and mediation of potent phagocyte migration and activation by its agonists suggest that FPRL1 shares many features of second-messenger activation with other G-protein-coupled chemoattractant receptors. In our previous study, incubation of human phagocytes with the FPRL1 agonist SAA resulted in a reduction of cell response to a number of chemoattractants (21), suggesting that activation of FPRL1 also initiates signaling events that desensitize other G-protein-coupled chemotactic receptors. In support of this hypothesis, we recently identified a peptide domain derived from HIV-1 gp120 that activated phagocytes preferentially through FPRL1 and down-regulated the expression and function of two chemokine receptors, CCR5 and CXCR4, which act as key fusion cofactors for HIV-1, through a protein kinase C-dependent pathway (22).

Our findings indicate the patho-physiological significance of the interaction between HIV-1 and phagocytic leukocytes. It has been reported that monocytes isolated from HIV-1-infected patients responded poorly to a variety of chemoattractants, including fMLF (13), *in vitro.* Several neutrophil functions are also impaired in HIV-infected patients, including chemotaxis, phagocytosis, oxidative metabolism, and killing of bacterial and fungal pathogens (23–28). However, the reason for reduced phagocyte function in such patients is not clear. We have found that recombinant soluble gp41 and gp120 of HIV-1 are able to potently down-regulate the expression and function of the receptors for a variety of chemotactic factors such as fMLF and a number of chemokines on monocytes, but not on neutrophils (11, 12). Soluble gp120 has been shown to also down-regulate the surface expression of the receptor for activated complement component C (C5a) on normal human monocytes (29). Mechanistic studies have shown that soluble gp120 and gp41 down-regulated chemoattractant receptors on monocytes by activating a protein kinase C (PKC)-mediated signaling pathway, a process resembling receptor "heterologous desensitization" (11, 12).

Our efforts to identify the structural basis for the effect of HIV-1 envelope proteins have yielded several peptide domains, such as T21/DP107 and T20/DP178, that are selective activators for the fMLF receptors FPR and/or FPRL1 (14, 17, 22). These findings, together with our present observation on the N36 peptide domain in gp41, suggest that HIV-1 envelope proteins contain multiple fragments that may potentially interact with cellular receptors, thus affecting the immune responses. Although the accessibility of such a HIV-1 envelope domain(s) *in vivo* to host immune cells remains to be determined, it has been reported that antibodies recognizing various epitopes in envelope proteins, such as T20/DP178 and T21/DP107, appear at early stages of HIV-1 infection (30–32). Therefore, even though formylated peptide receptors FPR and FPRL1 have not been reported as coreceptors used by HIV-1 for fusion, they may participate in the initial stimulation of the host innate defense responses seen in AIDS patients followed by a progressive inhibition of phagocytic cell function possibly due to desensitization of cell surface receptors. Recently, Munoz *et al.* (33) reported that superoxide production by neutrophils isolated from HIV-1-positive patients in response to stimulants of Fc receptor or PKC was significantly suppressed. Pretreatment of neutrophils, isolated from healthy donors, with a synthetic V2 domain derived

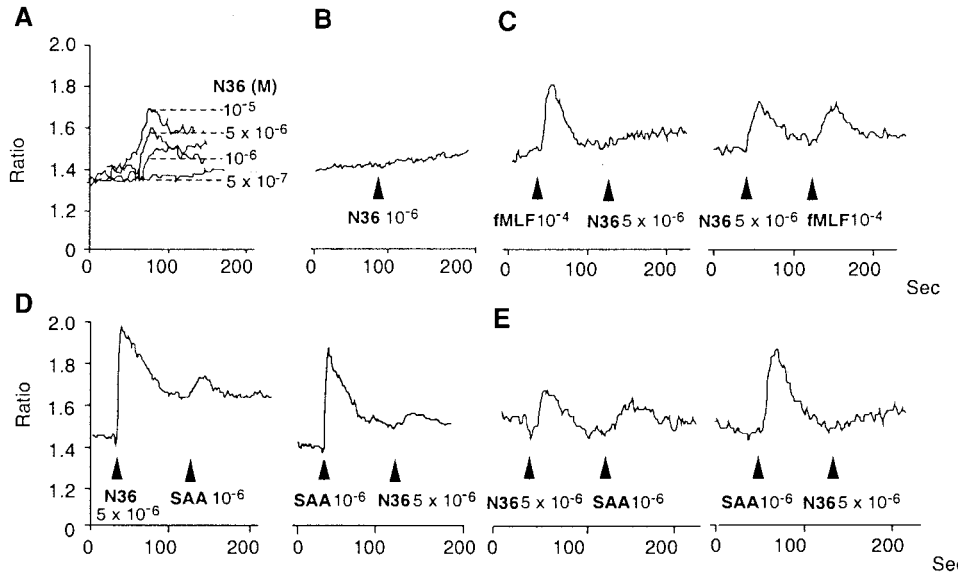

FIG. 5. Calcium mobilization induced by N36 peptide in FPRL1/293 cells and attenuation by fMLF or SAA. Cells were loaded with Fura-2. (A) FPRL1/293 cells were stimulated with different concentrations of N36 peptide. (B) N36 peptide did not induce calcium flux in ETFR cells. (C) Sequential stimulation of FPRL1/293 cells with N36 peptide and fMLF or vice versa. (D and E) Cross-desensitization of calcium mobilization between N36 and SAA in human monocytes (D) and FPRL1/293 cells (E).

from the HIV-1 SF2 strain envelope gp120 inhibited superoxide production of these cells in response to Fc receptor stimulants. Further studies showed that the V2 peptide reduced the phosphorylation of two cellular proteins, but increased the level of a third phosphorylated protein. These results may provide a basis for the observations that neutrophils from HIV-1-infected subjects exhibit "primed" phenotypes with reduced response to further stimulation (25, 28, 34, 35). Interestingly, while neutrophils are resistant to HIV-1 infection, eosinophils express HIV-1 coreceptors and are susceptible to viral infection (36). Eosinophils also express fMLF receptors FPR and FPRL1 (37). Further studies are required to elucidate the activity of HIV-1 envelope proteins and their domains on eosinophils and to determine whether these molecules contribute to the viral interference. The identification of domains in HIV-1 envelope proteins that activate phagocyte receptors may facilitate the understanding of the pathogenesis of AIDS and the development of immunoregulatory agents.

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