Synthetic Peptides of CD66a Stimulate Neutrophil Adhesion to Endothelial Cells

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Synthetic Peptides of CD66a Stimulate Neutrophil Adhesion to Endothelial Cells

Keith M. Skubitz,* Kenneth D. Campbell,* and Amy P. N. Skubitz†‡

Four members of the carcinoembryonic Ag family, CD66a, CD66b, CD66c, and CD66d, are expressed on human neutrophils. CD66a, CD66b, CD66c, and CD66d Ab binding to the neutrophil surface triggers an activation signal that regulates the adhesive activity of CD11/CD18, resulting in an increase in neutrophil adhesion to HUVEC. To identify active sites on the CD66a Ag, molecular modeling was performed using IgG and CD4 as models, and 28 peptides of 14 aa in length were synthesized that were predicted to be present at loops and turns between β-sheets. The peptides were tested for their ability to alter neutrophil adhesion to HUVEC. Three peptides, each from the N-terminal domain, increased neutrophil adhesion to HUVEC monolayers. This increase in neutrophil adhesion caused by CD66a peptides was associated with up-regulation of CD11/CD18 and down-regulation of CD62L on the neutrophil surface. Scrambled versions of these three peptides had no effect on neutrophil adhesion to the endothelial cells. The data suggest that peptide motifs from at least three regions of the N-terminal domain of CD66a are involved in the interaction of CD66a with other ligands and can initiate signal transduction in neutrophils. The Journal of Immunology, 2000, 164: 4257–4264.

CD66 family members appear to play a role in a wide variety of normal and pathological processes, including: cancer, embryonic development, bacterial infection, viral infection, inflammation, pregnancy, bile transport, and cell adhesion (1–3). CD66 mAbs react with members of the carcinoembryonic Ag (CEA) family (4–13). In the CD terminology, mAbs to CD66 mAbs react with members of the carcinoembryonic Ag (CEA) family (4–13). In the CD terminology, mAbs belonging to the CD66 cluster are classified according to their reactivity with each family member, as indicated by a lower case letter after “CD66” as follows: CD66a, CEA cell adhesion molecule (CEACAM)-1 or biliary glycoprotein; CD66b, CEACAM-8, or CGM6; CD66c, CEACAM-6, or NCA; CD66d, CEACAM-3, or CGM1; CD66e, CEA; and CD66f, pregnancy specific glycoprotein (13, 14). The CD66 (CEA) gene family belongs to the Ig gene superfamly (for review see Refs. 1, 2, and 15). Structurally, each of the human CD66 family members contains one amino-terminal (N) domain of 108–110 amino acid residues, homologous to Ig variable domains, followed by a differing number (0–6) of Ig C2-type constant-like domains. Therefore, the structure of the N-domain is predicted to be a stacked pair of β-sheets with nine β-strands (16).

CD66 family members may potentially function as adhesion molecules (12, 17–30). CD66a, CD66c, and CD66e are capable of homotypic and heterotypic adhesion, as shown by use of recombinant CD66a, which undergoes homotypic adhesion as well as heterotypic adhesion with CD66c and CD66e (1, 2, 4–12, 17–32). Data also suggest that CD66a plays a signaling role and regulates the adhesion activity of CD11/CD18 in human neutrophils (8, 11, 25–27, 33, 34). CD66a, CD66b, CD66c, and CD66d, but not CD66e, are expressed in human neutrophils, where they are “activation Ags” in that their surface expression is increased following neutrophil stimulation with various stimuli. CD66a, CD66b, CD66c, and CD66d mAb binding to the neutrophil surface triggers a transient activation signal that regulates the adhesive activity of CD11/CD18 and increases neutrophil adhesion to HUVECs (8, 11, 25–27, 33, 34). CD66a, the focus of this study, is frequently down-regulated in colon, prostate, breast, and hepatocellular carcinoma, and colorectal adenomas (35–39). Transfection studies have provided evidence that CD66a may act as a tumor suppressor in models of colon cancer (40), prostate cancer (41, 42), breast cancer (43), and bladder cancer (44). CD66a is also important in bacterial infections, because over 95% of pathogenic Neisseria meningitidis and Neisseria gonorrhoea interact with CD66a via Opa proteins, and the binding site for these Opa proteins has been localized to the N-domain of CD66a (45–50). An important property of Opa proteins is the stimulation of adhesion and nonopsonic phagocytosis of Opa+ bacteria by neutrophils (reviewed in Ref. 48). CD66a also appears to function as a receptor for murine hepatitis virus (51–55). Furthermore, CD66a may play a role in angiogenesis because it is selectively expressed on certain endothelial cells (56) and CD66a appears to function as a regulator of bile transport in bile canaliculi (3, 57, 58).

The mechanism(s) by which CD66a transmits signals (e.g., activation in neutrophils or growth regulating signals in epithelial cells and carcinomas) are unclear. However, CD66a is phosphorylated on its cytoplasmic domain, largely on tyrosine with a lower level of phosphoserine, in neutrophils and colon cancer cells (4, 59–61). While at least eight isoforms of CD66a derived from differential splicing have been described (3, 12, 13, 25), only those isoforms with a long cytoplasmic tail can be phosphorylated on tyrosine. In addition, associated protein tyrosine kinase and phosphatase activities may be involved in CD66a signaling (59, 62, 63).
Because of the adhesive and signaling properties of CD66a described above, we sought to identify functionally active domains of CD66a by use of synthetic peptides. To identify active sites on the extracellular domains of CD66a, molecular modeling was performed using IgG and CD4 as models. Twenty-eight peptides of 14 aa in length were synthesized that were predicted to contain loops and turns between β-sheets. Peptides were tested for their ability to alter neutrophil adhesion to HUVEC. Three peptides activated neutrophils, as determined by increasing neutrophil adhesion to HUVEC monolayers and altering surface expression of CD11/CD18 and CD62L. The data suggest that at least three peptide motifs from the N-terminal domain of CD66a are involved in the interaction of CD66a with other ligands and can initiate signal transduction in neutrophils.

Materials and Methods

Cell preparation

Normal peripheral blood neutrophils were prepared by a modification of the method of Boyum as previously described (64) and were suspended at the indicated concentrations in HBSS (Life Technologies, Grand Island, NY). Differential cell counts on Wright-stained cells routinely revealed >95% neutrophils. Viability as assessed by trypan blue dye exclusion was >98%.

Abs and reagents

The PE-labeled CD11b mAb (Leu 15) and the CD62L mAb (Leu 8) were obtained from Becton Dickinson (Mountain View, CA). Mononuclear Abs were diluted in PBS containing 1 mg/ml BSA as indicated. FMLP and normal mouse serum were purchased from Sigma (St. Louis, MO). Peptides were diluted in PBS containing 1 mg/ml BSA as indicated.

Fluorescence labeling of cells

Neutrophils were labeled with calcein AM (Molecular Probes, Eugene, OR) (65, 66) by incubating 5 × 10^6/ml cells with 50 μg of calcein AM for 30 min at 37°C in 18 ml of calcein labeling buffer (HBSS without Ca^{2+} or Mg^{2+} containing 0.02% BSA). Cells were then washed twice with calcein labeling buffer at 23°C and resuspended in the desired media.

Peptide selection, synthesis, and purification

CD66a was modeled to conform to the IgV and Ig C2 domains of the heavy and light chains of Fab of Ig and CD4. Our rationale was to select sequences from the loop regions of CD66a because these regions would potentially be more accessible to cells and therefore may be more biologically functionally active. CD66a has four Ig-like domains: one IgV-like domain termed the N domain at the amino terminal region, and three Ig C2-like domains termed the A1, B1, and A2 domains. When put into our model, the domains conformed to seven β-sheets and seven loop regions. Hydrophathy plots were also done using Kyte-Doolittle and Chou-Fasman analyses, and the results were consistent in that the chosen sequences co-incided with hydrophilic regions. The sequences were then standardized to a length of 14 amino acid residues, because this length results in a relatively high yield of peptides during synthesis. To complete this standardization, amino acid residues were added to the amino or carboxyl ends of the sequences. We also attempted to avoid having cysteine residues appear in the final sequences, because peptide dimerization may then occur and would complicate our assays. Our final selection of peptides to be synthesized are shown in Table I.

Some of the amino acids in the positive peptides, CD66a-1, CD66a-2, and CD66a-3, were randomly scrambled and the control peptides were synthesized (Table II). The scrambled amino acid residue peptides were then tested in the same assays to ensure that the primary amino acid sequences

<table>
<thead>
<tr>
<th>Table I. CD66a peptides^a</th>
<th>Peptide</th>
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<td>ISQKQSDPLMNVN</td>
<td>368–381</td>
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^a Peptides CD66a-1 to CD66a-7 are from the N domain, CD66a-9 to CD66a-14 are from the first C2-like domain, CD66a-16 to CD66a-20 are from the second C2-like domain, and CD66a-23 to CD66a-29 are from the third C2-like domain.

Peptides were synthesized as amides by F-moc solid-phase methodology on a Gilson Automated Multiple Peptide Synthesizer AMS 422 (Gilson, Middleton, WI). Peptides were purified by preparative reverse-phase HPLC on a Beckman System Gold (Beckman, Palo Alto, CA) equipped with a Regis Chemical ODS C18 column (10 μm particle size, 60 A pore size, 250 × 10 mm, particle size 5.1 μm). The elution gradient was 12–20% B over 35 min at a flow rate of 5.0 ml/min, where A was water containing 0.1% TFA and B was acetonitrile containing 0.1% TFA. Detection was at 235 nm. Peptides were analyzed for the correct amino acid composition by amino acid analysis, and all peptides were found to have the correct composition.

Once the peptides were screened in our adhesion assay (described below), the amino acids in the positive peptides, CD66a-1, CD66a-2, and CD66a-3, were randomly scrambled and the control peptides were synthesized (Table II). The scrambled amino acid residue peptides were then tested in the same assays to ensure that the primary amino acid sequences were essential for the functional activity of these peptides and that the biological activity was not merely due to the peptides' net charge or amino acid composition.

Endothelial cell adhesion assay

Neutrophil adhesion to HUVECs was determined as previously described (65–68). Briefly, HUVECs (Clonetics, San Diego, CA) were passaged 1:5 to 10–2 flask s (Costar, Cambridge, MA) no more than three times before plating in 96-well microwell plates at 3000 cells/well. HUVECs were grown to confluence in 96-well microwell plates in EGM media (Clonetics) and fed every 24 h. Using the adhesion assay described below, no difference in resting and stimulated neutrophil adhesion was observed, and, as expected (69), no difference in surface expression of CD54 (ICAM-1) or CD62E (E selectin, ELAM-1) in resting or TNF-stimulated cells was noted using HUVECs passaged once compared with those passaged five times. In some experiments, the HUVECs were stimulated by culture for the indicated time with the desired cytokines (TNF-α (Cetus, Emeryville, CA) or IFN-γ (a gift from Dr. S. Palm, University of Minnesota Medical School)). The wells were then washed four times with adhesion buffer (DMEM plus

<table>
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<tr>
<th>Table II. Scrambled peptides</th>
<th>Peptide</th>
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<td>CD66a-1</td>
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<td>CD66a-1-S1</td>
<td>LEXPVEMAPSNVG</td>
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<td>NPVEELMFGKAVS</td>
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<td>CD66a-1-S3</td>
<td>ENNPLSAFEVVKG</td>
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<td>CD66a-2</td>
<td>LVHIQLPQLGFSW</td>
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<td>CD66a-2-S1</td>
<td>QNLISLHFLFVPOQ</td>
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<td>CD66a-3-S3</td>
<td>GIVEYKGQVDNRHG</td>
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</table>
5% heat-inactivated FBS), and 25 μl of adhesion buffer containing the indicated peptide was added to each well, followed immediately by 100 μl of adhesion buffer containing 10^7 calcine-labeled cells. Then, 25 μl of adhesion buffer containing the indicated concentration of FMLP was added, and the plates were incubated at 37°C in 5% CO2 for 30 min. The wells were then aspirated and washed four times with endo wash buffer (HBSS plus 4% heat-inactivated FBS), and the fluorescence was quantitated with a Millipore fluorescence plate reader (Bedford, MA) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. For each condition, quadruplicate wells were tested and values are reported as the mean ± SD. Each experiment was performed at least four times using different HUVEC subcultures.

**Statistical analyses**

Effects of peptides on neutrophil adhesion to HUVECs was analyzed by the Mann Whitney U test when appropriate.

**Analysis of CD11b and CD62L expression**

For analysis of CD11b up-regulation, purified neutrophils (10^7 in 100 μl HBSS plus 0.02% BSA) were incubated with media containing the indicated peptide (167 μg/ml) or FMLP (10^{-7} M) for 15 min at 37°C. The cells were then cooled to 0°C for 10 min, and 2 μg of the PE-labeled CD11b mAb was added. The mixture was incubated at 0°C for 25 min, and 4 ml of buffer B (PBS, pH 7.4, 0.2% BSA, 0.05% NaN3) (0°C) was then added and the mixture was centrifuged at 400 × g for 5 min at 4°C. The supernatant was removed, the cells were vortexed and suspended in 1 ml of buffer B (0°C), and 250 μl of fixative (Coulter, Palo Alto, CA) (23°C) was then added. Then, 3 ml of buffer B (0°C) was added, and the mixture was centrifuged at 400 × g for 5 min at 4°C. The cells were washed with 3 ml of buffer B as above, resuspended in 200 μl of PBS containing 0.1% NaN3 (0°C), and stored at 4°C until analysis. Quantitative flow cytometric analysis of surface Ag expression was performed using a FACStar (Becton Dickinson). Forward and right angle light scatter, as well as the peak fluorescence channel, were optimized with fluorescent beads. The cell population was studied by forward and right angle light scatter.

For analysis of CD62L down-regulation, purified neutrophils (10^6 in 100 μl HBSS plus 0.02% BSA) were warmed to 37°C for 5 min and then incubated with media containing the indicated peptide (167 μg/ml) or FMLP (10^{-7} M) for 5 min at 37°C. The cells were then cooled to 0°C for 10 min, and 5 μg of the PE-labeled CD62L mAb was added. The cells were then incubated, washed, and analyzed by flow cytometry as above.

**Results**

**Synthesis of CD66a peptides**

Because CD66a is a member of the Ig superfamily, we modeled CD66a using the known crystallographically determined structure of the IgV and Ig C2-like domains of IgG and CD4. The amino acid sequence of CD66a was also analyzed by a hydropathy plot using Kyte-Doolittle and Chou-Fassman analyses, and sequences predicted to be exposed on the surface of the molecules based on hydrophilicity were identified. There was good agreement in general between the peptides selected using these two methods. A hydrophilicity was identified. There was good agreement in general between the peptides selected using these two methods. A general between the peptides selected using these two methods.

**Effects of CD66a peptides on neutrophil adhesion to endothelial cells**

The CD66a peptides were tested for their ability to alter neutrophil adhesion to HUVECs stimulated for 48 h with 1000 U/ml IFN-γ and 50 ng/ml TNF-α (Fig. 1). When neutrophils were incubated for 30 min in the presence of media containing 167 μg/ml of each peptide with these HUVECs, and washed as described in Materials and Methods, three peptides (CD66a peptides CD66a-1, CD66a-2, and CD66a-3) augmented neutrophil adhesion ~2-fold compared with media (Fig. 1, solid bars). This effect was more prominent in the presence of 10^{-7} M FMLP (hatched bars). In contrast, the other peptides did not alter neutrophil adhesion when compared with incubation in media alone. Similar results were obtained using HUVECs stimulated for 4 h with 50 μg/ml TNF-α (not shown).

The three peptides that specifically induced neutrophil adhesion were further tested for their effects on the adhesion of neutrophils to TNF-stimulated HUVECs. Each of the three CD66a peptides, CD66a-1, CD66a-2, and CD66a-3, increased neutrophil adhesion to HUVECs at concentrations as low as 50 μg/ml (~35 μM) in the presence of FMLP (Fig. 2). To confirm that the activity of these peptides was due to the sequence and not simply a charge effect, three scrambled versions were made of each active peptide (Table II) and tested in the adhesion assay. In contrast to the native peptides, none of the nine scrambled peptides had activity in the adhesion assay (Fig. 3).

To test the possible role of stimulation of HUVECs by peptides, the peptides were added to HUVECs for 30 min and then the HUVECs were washed before adding neutrophils. This experiment did not detect activation of the HUVECs by any of the peptides (data not shown). However, due to the time required for manipulation, we could not exclude a contribution of peptide-mediated HUVEC stimulation that required the presence of neutrophils. Studies were also performed to test for possible synergistic effects of the three active peptides (CD66a-1, CD66a-2, and CD66a-3). No evidence of synergy was detected in the presence or absence of FMLP (data not shown).

**Effect of CD66a peptides on CD11b expression**

The effects of the peptides on surface expression of CD11b on neutrophils was next examined. While neutrophil adhesion to HUVECs is dependent on the functional activity of surface CD11/
Effects of scavenged CD66a peptides on neutrophil adhesion to HUVECs. HUVECs were grown to confluence in 96-well microtiter plates and stimulated by incubating in the presence of TNF-α at 50 ng/ml final concentration for 4 h at 37°C, and the adhesion of neutrophils was quantitated in the presence of the indicated final concentration of CD66a peptide CD66a-1 (○), CD66a-2 (□), or CD66a-3 (△) and 10⁻⁷ M FMLP as described in Fig. 1. Values are shown as the percent of added neutrophils remaining adherent to the HUVEC monolayers and represent the means ± SD of four separate determinations. The adhesion observed in the presence of CD66a peptides CD66a-1, CD66a-2, and CD66a-3 at 50 µg/ml was statistically greater than that observed with lower concentrations of peptides (p < 0.05).

Effects of scavenged CD66a peptides on neutrophil adhesion to HUVECs. HUVECs were grown to confluence in 96-well microtiter plates and stimulated by incubating in the presence of TNF-α at 50 ng/ml final concentration for 4 h at 37°C, and the adhesion of neutrophils was quantitated in the presence of the indicated final concentration of CD66a peptide CD66a-1 (○), CD66a-2 (□), or CD66a-3 (△) and 10⁻⁷ M FMLP as described in Fig. 1. Values are shown as the percent of added neutrophils remaining adherent to the HUVEC monolayers and represent the means ± SD of four separate determinations. The adhesion observed in the presence of CD66a peptides CD66a-1, CD66a-2, and CD66a-3 at 50 µg/ml was statistically greater than that observed with lower concentrations of peptides (p < 0.05).

FIGURE 3. Effects of scavenged CD66a peptides on neutrophil adhesion to HUVECs. HUVECs were grown to confluence in 96-well microtiter plates and stimulated by incubating in the presence of TNF-α at 50 ng/ml final concentration for 4 h at 37°C. The wells were then washed, and 25 µl of adhesion buffer containing the indicated CD66a peptides (at 167 µg/ml final concentration) was added. Then, 100 µl of adhesion media containing 10⁵ neutrophils was added, followed by 25 µl of adhesion buffer without peptide or with (■) 6 × 10⁻⁷ M FMLP, and the plates were incubated at 37°C for 30 min in 5% CO₂. The wells were then washed, and the number of adherent neutrophils was determined with a fluorescence plate reader as in Fig. 1. Values are shown as the percent of added neutrophils remaining adherent to the HUVEC monolayers and represent the means ± SD of four separate determinations. The adhesion observed in the presence of the active CD66a peptides CD66a-1, CD66a-2, and CD66a-3 was statistically greater than that observed with the nine scrambled peptides (p < 0.05).

FIGURE 4. Representative flow cytometric histogram profiles of the effect of scavenged CD66a peptides on human neutrophil surface CD11b and CD62L expression. Left, Purified neutrophils were incubated with HBSS (MCF = 584) (top panel), FMLP (10⁻⁷ M) (MCF = 709) (second panel), the CD66a peptide CD66a-1 (MCF = 704) (167 µg/ml) (third panel), the CD66a peptide CD66a-2 (MCF = 713) (167 µg/ml) (fourth panel), the CD66a peptide CD66a-3 (MCF = 714) (167 µg/ml) (fifth panel), or the scrambled CD66a peptide CD66a-1-S1 (MCF = 581) (167 µg/ml) (bottom panel) for 15 min at 37°C, and the binding of a PE-labeled CD11b mAb was determined as described in Materials and Methods. Vertical axis, relative cell number; horizontal axis, relative fluorescence intensity measured on a log scale. The MCFs represent the means of two determinations that agreed within 10%. Right, Purified neutrophils were warmed to 37°C, incubated for 5 min with HBSS (MCF = 548) (top panel), FMLP (10⁻⁷ M) (MCF = 256) (second panel), the CD66a peptide CD66a-1 (MCF = 230) (167 µg/ml) (third panel), the CD66a peptide CD66a-2 (MCF = 243) (167 µg/ml) (fourth panel), the CD66a peptide CD66a-3 (MCF = 229) (167 µg/ml) (fifth panel), or the scrambled CD66a peptide CD66a-1-S1 (MCF = 546) (167 µg/ml) (bottom panel), and the binding of a PE-labeled CD62L mAb was determined as described in Materials and Methods. Vertical axis, relative cell number; horizontal axis, relative fluorescence intensity measured on a log scale. A duplicate experiment gave similar results.

Effect of scavenged CD66a peptides on neutrophil adhesion, CD11b expression was analyzed by flow cytometry. Because CD11 and CD18 are translocated to the cell surface only when they are complexed with each other, the use of a directly labeled CD11b mAb was used to demonstrate up-regulation of CD18 as well as CD11b. When neutrophils were incubated with HBSS for 15 min at 37°C and then reacted with a PE-labeled CD11b mAb, CD11b expression was readily detected by flow cytometry (mean channel fluorescence (MCF) = 584) (Fig. 4, top panel). As expected, when neutrophils were incubated with FMLP (10⁻⁷ M) for 15 min, CD11b expression was increased (MCF = 709) (Fig. 4, second panel). When neutrophils were incubated with 167 µg/ml of the CD66a peptide CD66a-1 (MCF = 704) (Fig. 4, third panel), the CD66a peptide CD66a-2 (MCF = 713) (Fig. 4, fourth panel), or the CD66a peptide CD66a-3 (MCF = 714) (Fig. 4, fifth panel), CD11b expression also increased, similar to that seen with incubation with 10⁻⁷ M FMLP. In contrast, incubation with the scrambled CD66a peptide CD66a-1-S1 resulted in similar CD11b expression as incubation with HBSS (MCF = 581) (Fig. 4, bottom panel), as did the other eight scrambled peptides (not shown).
Effect of CD66a peptides on CD62L expression

The effects of the peptides on surface expression of CD62L on neutrophils was next examined. L-selectin, recognized by CD62L mAbs, also plays a role in neutrophil adhesion to endothelial cells, and its expression is altered by stimulation (70, 72). To determine whether the surface expression of CD62L could be altered by CD66a peptides, CD62L expression was analyzed by flow cytometry. When neutrophils were incubated with HBSS for 5 min at 37°C, and then reacted with a PE-labeled CD62L mAb, CD62L expression was readily detected by flow cytometry (MCF = 548) (Fig. 4, top panel). When neutrophils were incubated with 10−7 M FMLP, CD62L expression decreased as expected (MCF = 256) (Fig. 4, second panel). Similarly, when neutrophils were incubated with the CD66a peptide CD66a-1, (MCF = 230) (Fig. 4, third panel), the CD66a peptide CD66a-2 (MCF = 243) (Fig. 4, fourth panel), or the CD66a peptide CD66a-3 (MCF = 229) (Fig. 4, fifth panel), CD62L expression also decreased. Incubation with the scrambled CD66a peptide CD66a-1-S1 did not alter CD62L expression (MCF = 546) (Fig. 4, bottom panel). Similarly, none of the other eight scrambled peptides altered CD62L expression (not shown).

Discussion

Twenty-eight peptides were synthesized from regions of CD66a that we predict form loops and turns between regions of β-sheets and may be exposed on the surface of the molecule. Three of the peptides were found to have activity in an assay examining stimulated neutrophil adhesion to HUVECs. These same three peptides also stimulated up-regulation of CD11b/CD18 and down-regulation of CD62L on the neutrophil surface. Scrambled versions of these peptides had no biological activity in either assay, suggesting that the specific amino acid sequence is critical for activity. Thus, the data suggest that peptide motifs from at least three regions of the N-terminal domain of CD66a are involved in the interaction of CD66a with other ligands and can initiate signal transduction in neutrophils.

Several other studies have proposed structural motifs of CD66a family proteins (16, 21, 73). While our peptides of 14 amino acid residues in length contain variable numbers of residues from proposed β-sheet regions, our model is in general similar to other reported models.

All three active peptides identified in this study are derived from the N-terminal domain of CD66a. Studies of transfectants and recombinant proteins have suggested that the N-terminal domain is critical for the homotypic and heterotypic adhesion activity of CD66a (12, 21, 23, 25, 32). Studies using domain-specific mAbs have also suggested that the N-domains of CD66 family members are important in homotypic adhesion (21, 24). However, studies have also suggested that the A1, B1, or A2 domains may also be important in homotypic adhesion, and may interact with the N-domain (12, 19, 20, 22, 23).

Although carbohydrates on CD66 family members may play important roles, the protein backbone itself appears to have important activity in this and other studies. For example, bacterial fusion proteins free of carbohydrates containing the N or A3B3 domains of CD66e can block CD66e homotypic adhesion, demonstrating that protein-protein interaction is involved in CD66e homotypic adhesion (23). Deglycosylated forms of CD66b and CD66c retain heterotypic adhesion activity (31), further demonstrating that carbohydrates are not necessary for their adhesion functions. In addition, both recombinant N-terminal domains of CD66a and CD66e expressed in Escherichia coli bind Opa proteins with the same specificities as native CD66 molecules, and deglycosylated forms of CD66e bind bacterial Opa proteins (50).

Site-directed mutagenesis studies of the related proteins C-CAM-105 and CEA (CD66e) have identified regions important for certain functional activities. For example, the integrity of Arg98 in the consensus ATPase domain (GPAYSGRET) of C-CAM-105 is essential for homotypic aggregation (58). This arginine is highly conserved in Ig domains, being important in forming a salt bridge with a highly conserved aspartate within the same domain (16). Our model predicts that the consensus ATPase domain is present in a loop/turn region comprising the sequence of peptide CD66a-5. However, peptide CD66a-5 had no activity in our assay.

The finding that these short peptides can stimulate neutrophils, as can CD66a mAbs (26–28, 67, 74, 75), suggests that they have significant affinity for a surface structure, possibly native CD66a. If so, whether the activity derives from binding native CD66a and transducing a signal directly or by another mechanism will require further study. The ability of the synthetic peptides described here to activate neutrophils could be mediated by alterations in CD66a dimerization, possibly by disrupting a preexisting association of CD66a with other CD66 members (including CD66a itself in the form of dimers or oligomers already present on the cell surface) or by stimulating dimerization. It has been suggested that CD66a (76) and CD66e (77) exist on the cell surface as dimers. Dimerization of CD66a could potentially occur via interactions between the extracellular domains of CD66a molecules or via other mechanisms. In other receptor systems (e.g., monomeric epidermal growth factor, dimeric platelet-derived growth factor), it is clear that bivalency of ligand is not necessary to induce receptor dimerization (78–81). Finally, the observed functional “stimulation” could reflect either “stimulation” per se or possibly release from a baseline inhibition.

The mechanisms by which CD66 family members transmit signals (e.g., activation in neutrophils, immune suppression of lymphocytes, or growth regulating signals in epithelial cells and carcinomas) are unclear. CD66a is phosphorylated in neutrophils and colon cancer cells (4, 59–61), and associated protein kinase and phosphatase activity may be involved (59, 62). At least eight isoforms of CD66a derived from differential splicing have been described (3, 12, 13, 25). These isoforms contain one N domain, either three, two, or no Ig C2-like domains, and either a short or a long cytoplasmic tail. Only those isoforms with a long cytoplasmic tail can be phosphorylated on tyrosine, and only the isoform with four Ig domains and a long cytoplasmic tail (the only isoform detected in neutrophils) have been implicated in signaling. The cytoplasmic domain of neutrophil CD66a contains an immune tyrosine inhibitory motif (VXXYYL), as well as a motif similar to immune tyrosine activating motif (3, 59). Phosphorylation of immune tyrosine inhibitory and activating motifs leads to binding of protein tyrosine kinases and protein tyrosine phosphatases, respectively, which leads to modification of signal transduction (62, 63). Calmodulin has also been found to bind to the cytoplasmic domain of CD66a, causing an inhibition of homotypic self-association of CD66a in a dot-blot assay (82). CD66a has also recently been shown to dimerize in solution, and calcium-activated calmodulin caused dissociation of CD66a dimers in vitro; suggesting that CD66a dimerization is regulated by calmodulin and intracellular calcium (76). It has been suggested that CD66a dimerization could also be influenced by phosphorylation; CD66a is phosphorylated on Thr453 in the calmodulin binding site by protein kinase C (3). Clearly, dimerization of CD66a could affect binding of other signal regulating molecules.

CD66 family members appear to be involved in a wide variety of important biological processes, and their differential expression provides the possibility for diverse interactions. For example, CD66a, CD66b, CD66c, and CD66d, but not CD66e, are expressed on neutrophils; CD66e is expressed on many tumor cells but not...
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