Domain-specific Interactions between Entactin and Neutrophil Integrins

G2 Domain Ligation of Integrin α5β1 and E Domain Ligation of the Leukocyte Response Integrin Signal for Different Responses*

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Extracellular matrix proteins activate neutrophils to up-regulate many physiologic functions that are necessary at sites of tissue injury. To elucidate the ligand-receptor interactions that mediate these functions, we examined neutrophil activation by the basement membrane protein, entactin. Entactin is structurally and functionally organized into distinct domains; therefore, we utilized glutathione S-transferase -fusion proteins encompassing its four major domains, G1, G2, E, and G3, to assess interactions between entactin and neutrophil integrin receptors. We show that the E domain, which contains the single RGD sequence of entactin, is sufficient for ligation of the β3-like integrin, leukocyte response integrin, and signaling for chemotaxis. Moreover, the G2 domain signals for stimulation of Fc receptor-mediated phagocytosis via ligation of α5β1. This receptor-ligand interaction was revealed only after stimulation of neutrophil by immune complexes or phorbol esters. Interestingly, the E domain does not enhance phagocytosis, and the G2 domain is not chemotactic. Furthermore, cleavage of entactin with the matrix metalloproteinase, matrilysin, liberates peptides that retain E domain-mediated chemotaxis and G2 domain-mediated enhancement of phagocytosis. These studies indicate that multiple domains of entactin have the ability to ligate individual integrins expressed by neutrophils and to activate distinct functions.

Polymorphonuclear neutrophils (PMNs) circulate in the vasculature in a quiescent state and undergo a number of important functional changes during recruitment to a site of inflammation or infection. Extracellular matrix proteins and peptides derived from them activate PMN to up-regulate many of these physiologic functions, including adhesion, migration, generation of reactive oxygen intermediates, and increased phagocytosis of opsonized pathogens (1–10). Understanding the ligand-receptor interactions and intracellular signals involved when PMNs encounter matrix or adhesive proteins is essential in order to develop therapeutic interventions that either enhance desirable or inhibit deleterious effects of PMN activation at sites of tissue injury.

To further understand the receptor-ligand interactions that contribute to PMN activation by matrix proteins, we have initiated studies examining PMN activation by the basement membrane protein entactin (nidogen) (7). Entactin is a 150-kDa sulfated glycoprotein comprised of several structurally and functionally distinct domains (11). It consists of three globular regions and a rigid stalk that together generate an asymmetric dumbbell-like structure (12). The amino-terminal globular regions (G1 and G2) are linked to each other by a single EGF-like repeat and are connected to the carboxy-terminal globular domain (G3) by the cysteine-rich stalk (E domain), which consists of 4 EGF-like repeats and a single thyroglobulin-like repeat (13). Two cell adhesion sites have been defined in entactin: one is comprised of the first EGF repeat in the E domain and contains an Arg-Gly-Asp (RGD) sequence, and the second is comprised of the EGF-like repeat flanking the amino terminus of the G2 domain (14). The integrin α5β3 recognizes the RGD sequence within the E domain (14), and α5β1 recognizes the G2 domain (15). Whereas both of these integrins appear to mediate cell attachment to entactin, it is not known how these receptors may interact or if they mediate distinct signals for cell activation following ligation by the different domains within entactin.

Our previous studies indicated that ligation of the β3-like leukocyte response integrin (LRI) by the RGD sequence within entactin in conjunction with an associated protein, integrin-associated protein (IAP, CD47), promoted PMN adhesion and chemotaxis (7). To extend this observation, we used GST-fusion proteins encompassing the four domains of entactin to assess integrin ligation and PMN activation. We report here that PMNs recognize at least two distinct sites within entactin, which activate distinct PMN responses, that the ligand binding site for one of these sites is revealed only after activation of PMN by immune complexes and chemotactic peptides, and that these separate sites were retained after cleavage of entactin by the matrix metalloproteinase, matrilysin, indicating that pro-

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¶ The abbreviations used are: PMN, polymorphonuclear neutrophil; E1G1G, IgG-opsonized erythrocytes; GST, glutathione-S-transferase; hpg, high power grid; HSA, human serum albumin; HBSS, Hanks’ balanced salt solution; BSA, bovine serum albumin; IAP, integrin-associated protein; LRI, leukocyte response integrin; PDBu, phorbol 12,13-dibutyrate; MLLP, N-formyl-methionyl-leucyl-phenylalanine; mAb, monoclonal antibody; CHO, Chinese hamster ovary; EGF, epidermal growth factor.

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Entactin Domains and PMN Activation

Entactin domains present at inflammatory sites could liberate these functional domains from tissue bound matrix. These studies indicate that multiple domains of entactin have the ability to ligate individual integrins expressed by PMN and to activate distinct PMN functions.

**EXPERIMENTAL PROCEDURES**

**Reagents—**N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12,13-dibutyrate (PDBu), and catalase (bovine liver, 52,000 units/mg) were purchased from Sigma. fMLP (22.9 mM) and PDBu (1 mg/ml) were prepared as stock solutions in Me2SO (Aldrich), stored at −70 °C, and diluted into aqueous medium immediately before use. Equivalent concentrations of vehicle were used to control for solvent effects. Pyrogen-free water for buffer preparation was obtained from Kendall McGaw, Irvine, CA. Purified rabbit IgG anti-bovine serum albumin (BSA) and purified BSA were purchased from Organon Teknika Corp., West Chester, PA.

Entactin Domains and Fusion Proteins—

Murine entactin was purified from M1536-B3 extracellular matrix, and recombinant murine entactin was obtained from a baculovirus expression system as described (19). Recombinant entactin digested by matrixysis was prepared as previously described (20). Briefly, 0.88 μg of recombinant matrixysis, which had been activated with aminophenylisocyanate and was incubated with 89 μg of entactin at 37 °C for 15 min. A mutant entactin in which aspartic acid at residue 674 was replaced with glutamic acid (RGE-entactin) was prepared as previously described (7). Using similar procedures, a second mutant entactin was made that lacked the RGD sequence (ΔRGD-entactin). The oligonucleotide complementary to the coding sequence that was employed for this deletion was 5′-ATC GTA AGT AGT CGT CCC GAA GCC GAT GGA GCA TTC-3′. This oligonucleotide lacked the nine bases complementary to nucleotides 2109−2117 that encoded the RGD sequence in entactin and had eighteen matching bases flanking each side of the deleted sequence. The deletion was confirmed by DNA sequencing of the mutant construct. GST-fusion proteins consisting of GST fused to the G1, G2, and E domains of entactin were constructed and purified as previously described (14, 21). The amino acid sequences of the respective domains consisted of residues 7−301 (G1), residues 301−647 (G2), and residues 639−893 (E).

**Cells—**PMN were isolated from normal volunteers without hypotonic lysis of the contaminating erythrocytes to prevent possible damage to the PMN as previously described (17). Platelet counts were performed on the PMN preparations and were less than 10,000 platelets/100 PMN. PMN were suspended in HBSS containing 4.2 mM NaHCO3, 1 mM EDTA to help disrupt the aggregates and then washed twice in the incubating medium immediately before use. Without washing, the mixtures were incubated with 45 μM of either the HSA-entactin, ΔRGD-entactin, G1 domain-, G2 domain-, or E domain-coated beads in the presence of one of the following stimuli as indicated in the above figure legends: 200 μg/ml of immune complexes (BSA-anti-BSA, 10−3 of fMLP, 30 mM PDBu, 200 mM PDBu, or vehicle control. After 1 h at 37 °C, adhesion was assessed by fluorescence microscopy and quantified as an attachment index, the number of beads bound by 100 cells. Preliminary experiments assessing the optimal divalent cation concentrations for binding of the various coated beads were performed. Where β1 integrins were primarily involved, optimal binding was obtained with 1.0 mM Mg2+, and where LRI was involved, optimal binding was obtained with 0.5 mM Ca2+ and 2.5 mM Mg2+. The divalent cation concentration of the buffer is indicated in the above figure legends. In all experiments, qualitatively similar data were obtained in buffer containing 1.5 mM Ca2+ and 1.5 mM Mg2+.

**RESULTS**

**Entactin Stimulates Phagocytosis of EIgG via αββ1 and Not via LRI and IAP—**To pursue our studies on the interaction of PMN with entactin, we examined the ability of entactin to stimulate ingestion of EIgG. We chose amplification of Fc receptor-mediated phagocytosis because we had shown previously that other matrix and adhesive proteins stimulated IgG-dependent phagocytosis via LRI and IAP (5, 6) and that entactin promoted PMN chemotaxis via LRI and IAP (7). Recombinant entactin stimulated ingestion of EIgG in a dose-dependent fashion with an optimal concentration at 10 μg/ml (Fig. 1, A and B), and as with other stimuli that enhance Fc receptor-mediated ingestion, the dose-response curve was biphasic (5, 6, 17, and 18). This increase in phagocytic index reflected both an increase in the percentage of PMN ingesting EIgG and in the number of EIgG ingested by each PMN. To our surprise, inhibitory mAb against either LRI or IAP had no effect on entactin-stimulated ingestion (Fig. 1, A and B). Because the integrin αββ1 had been implicated in other adhesive functions of entactin (24), we examined the effect of mAbs against β1 and αβ. Treatment of PMN with either of these mAbs completely abrogated entactin-stimulated ingestion (Fig. 1, C and D). Another mAb against αβ (BD) also resulted in inhibition (data not shown). An isotype control mAb had no effect (Fig. 1A). Because both the anti-β1 and -αβ mAbs were used as a 1:50 dilution of ascites and could be inhibiting nonspecifically, we...
assessed the effect of mAb anti-α3 as a 1:5 dilution of ascites. This mAb, which recognizes an integrin also expressed by PMN, β1 (5), had no effect (Fig. 1B). To further control for nonspecific effects, we assessed the ability of P4C10 and 7G2 to inhibit ingestion stimulated by the phorbolester PDBu, and neither mAb had any effect on PDBu-stimulated ingestion (data not shown). Therefore, the ability of the mAbs against α3 and β1 to inhibit entactin-stimulated ingestion of IgG3 was specific. These data indicate that entactin stimulates Fc receptor-mediated phagocytosis via α3β1 and not via LRI and IAP.

Because these data were obtained with recombinant entactin that is denatured during purification, we examined the ability of native entactin to stimulate ingestion of IgG in an α3β1-dependent fashion, and it enhanced phagocytosis in a manner indistinguishable from that seen with recombinant entactin (data not shown). Moreover, mAb anti-α3 completely abrogated the ability of native entactin to augment phagocytosis as compared with a control antibody. These data indicate that the ability of entactin to promote IgG-dependent ingestion via α3β1 can be observed with both recombinant and native entactin.

**PMN Activation Changes the Binding of Entactin from LRI to α3β1.—**To ascertain if the inhibitory effects of anti-β1 and -α3 on entactin-stimulated phagocytosis reflected an inhibition of ligand binding to the integrin, we assessed the binding of entactin-coated fluorescent beads to PMN in the presence and absence of immune complexes to mimic Fc receptor stimulation provided by the IgG-opsonized sheep erythrocytes during the phagocytosis assay. In the absence of immune complex stimulation, binding of entactin beads to PMN was reduced by both anti-LRI and -IAP to the level of binding observed with control HSA-coated beads, indicating that LRI and IAP were primarily responsible for entactin binding to unstimulated PMN (Fig. 2). In contrast, anti-α3 had no effect (Fig. 2). With immune complex stimulation, the level of entactin-head binding increased 3–4 fold over that observed in the absence of stimulation. Moreover, anti-LRI and -IAP had no effect on this binding. In contrast, anti-α3 significantly inhibited entactin-bead binding to stimulated PMN, indicating that α3β1 is primarily responsible for the binding of entactin to activated PMN (Fig. 2). Qualitatively and quantitatively similar data were obtained when

**FIG. 1. Effect of recombinant entactin on phagocytosis of IgG by PMN in the presence of: buffer, IgG1 mAb control (2D3), IgG1 mAb anti-LRI (7G2), or IgG1 mAb anti-β1 (P4C10) (A), and IgG1 mAb anti-IAP (B6H12), IgG1 mAb anti-α3 (P1B5), or IgG3 mAb anti-α3 (P1D6) (B).** PMNs (1.0 × 10⁶) were incubated with 7G2 (0.25 µg), B6H12 (0.25 µg), P4C10 (1:50 dilution of ascites), P1B5 (1:50 dilution of ascites), P1D6 (1:50 dilution of ascites), or control antibody 2D3 (0.25 µg) in the presence of 5,000 units of catalase for 15 min at room temperature. Without washing, the mixtures were incubated with IgG3 in the presence of buffer or the indicated concentrations of entactin in a final volume of 1.15 ml. After 30 min at 37°C, ingestion was assessed. Phagocytic index = number of IgG ingested by 100 PMNs. Data are representative of four experiments. Antibodies against α3β1, but not against LRI and IAP, inhibited entactin-stimulated Fc receptor-mediated phagocytosis.

PMNs were stimulated with either phorbolester (30 nM PDBu) or a chemotactic peptide (1 × 10⁻⁷ m FMLP) (data not shown). These data indicate that PMN activation, which occurs as a result of Fc receptor stimulation, changes the binding site for entactin on PMN from LRI to α3β1.

**The Ability of Entactin to Stimulate Ingestion and to Bind to Activated PMN via α3β1 Is Independent of the RGD Sequence of Entactin—**Because previous data indicated that the RGD sequence within entactin was essential to promote PMN adhesion and chemotaxis (7) and that α3β1 could recognize RGD sequences in some ligands (23), we assessed the ability of entactin, where its RGD sequence at 672–674 has been deleted (24), to augment PMN ingestion of IgG and to bind to immune complex-stimulated PMN. RGD-entactin was fully capable of augmenting ingestion of IgG and inclusions of either anti-α3 or -β1, as compared with an isotype control antibody, abrogated the enhanced ingestion (Fig. 3A). Similar data were obtained with RGE-entactin (data not shown). These data indicate that ligation of α3β1 by ΔRGD-entactin is fully capable of signaling for augmentation of ingestion.

When the binding of ΔRGD-entactin-coated fluorescent beads to unstimulated PMN was assessed, the data were significantly different from those obtained in Fig. 2 for entactin-coated bead binding to unstimulated PMN. First, the binding of ΔRGD-entactin-coated beads was not inhibited by either anti-LRI or -IAP (Fig. 3B), whereas these antibodies did inhibit the binding of entactin-coated beads (Fig. 2). These data confirm our previous observation that the RGD sequence within entactin is essential for entactin-mediated ligation of LRI (7). Second, inclusion of antibodies against β1 and α3 enhanced the binding of the ΔRGD-entactin-coated beads. The ability of some antibodies against β1 to promote integrin ligand binding has been observed by others (25–27). In contrast, when PMNs were stimulated with immune complexes, the binding of the ΔRGD-entactin-coated beads was inhibited by the inclusion of anti-β1.
entactin, with its RGD sequence deleted (ARGD-entactin), to stimulate phagocytosis of ElG by PMN via $\alpha_3\beta_1$ (A) and to bind to PMN stimulated with immune complexes via $\alpha_4\beta_1$ (B). A, PMNs (1.0 x 10$^6$) were incubated with anti-$\alpha_3$ (P1B5), 1:50 dilution of ascites), anti-$\beta_1$ (4B4, 6 $\mu$g), or control murine IgG1 (6 $\mu$g) in the presence of 5,000 units of catalase for 15 min at room temperature. Without washing, the mixtures were incubated with ElG in the presence of buffer or the indicated concentrations of entactin in a final volume of 115 $\mu$L. After 30 min at 37°C, ingestion was assessed. Phagocytic index = number of ElG ingested by 100 PMNs. ARGD-entactin was fully capable of augmenting Fc receptor-mediated phagocytosis, and this augmentation requires the integrin, $\alpha_3\beta_1$, B, binding of $\Delta$RGD-entactin-coated fluorescent beads to PMN in the presence of buffer (HBSS containing 1% HSA with 1 mM Mn$^{2+}$) or immune complexes (rabbit IgG anti-BSA, BSA) was assessed as described in the legend for Fig. 3. The effect of the following antibodies was measured: anti-$\alpha_3$ (P1B5), $\beta_1$ (A1A5, 4B4), LRI (7G2), IAP (B6H12), or a murine IgG1 control. The binding of HSA-coated beads was used to assess nonspecific binding. Data are depicted as the mean $\pm$ S.E., n = 3. Binding of beads coated with ARGD-entactin to PMN in buffer was not inhibited by antibodies against LRI, IAP, or $\alpha_3\beta_1$. In contrast, binding of $\Delta$RGD-entactin-coated beads to immune complex-stimulated PMN was inhibited by antibodies against $\alpha_3\beta_1$ but not by antibodies against LRI or IAP. Therefore, binding of entactin to stimulated PMN via $\alpha_3\beta_1$ did not require the RGD sequence of entactin.

Fig. 3. Ability of entactin, with its RGD sequence deleted (ARGD-entactin), to stimulate phagocytosis of ElG by PMN via $\alpha_3\beta_1$ (A) and to bind to PMN stimulated with immune complexes via $\alpha_4\beta_1$ (B). A, PMNs (1.0 x 10$^6$) were incubated with anti-$\alpha_3$ (P1B5), 1:50 dilution of ascites), anti-$\beta_1$ (4B4, 6 $\mu$g), or control murine IgG1 (6 $\mu$g) in the presence of 5,000 units of catalase for 15 min at room temperature. Without washing, the mixtures were incubated with ElG in the presence of buffer or the indicated concentrations of entactin in a final volume of 115 $\mu$L. After 30 min at 37°C, ingestion was assessed. Phagocytic index = number of ElG ingested by 100 PMNs. ARGD-entactin was fully capable of augmenting Fc receptor-mediated phagocytosis, and this augmentation requires the integrin, $\alpha_3\beta_1$, B, binding of $\Delta$RGD-entactin-coated fluorescent beads to PMN in the presence of buffer (HBSS containing 1% HSA with 1 mM Mn$^{2+}$) or immune complexes (rabbit IgG anti-BSA, BSA) was assessed as described in the legend for Fig. 3. The effect of the following antibodies was measured: anti-$\alpha_3$ (P1B5), $\beta_1$ (A1A5, 4B4), LRI (7G2), IAP (B6H12), or a murine IgG1 control. The binding of HSA-coated beads was used to assess nonspecific binding. Data are depicted as the mean $\pm$ S.E., n = 3. Binding of beads coated with ARGD-entactin to PMN in buffer was not inhibited by antibodies against LRI, IAP, or $\alpha_3\beta_1$. In contrast, binding of $\Delta$RGD-entactin-coated beads to immune complex-stimulated PMN was inhibited by antibodies against $\alpha_3\beta_1$ but not by antibodies against LRI or IAP. Therefore, binding of entactin to stimulated PMN via $\alpha_3\beta_1$ did not require the RGD sequence of entactin.

and $\alpha_3$ (Fig. 3B). These data were similar to those obtained in Fig. 3 for the effect of anti-$\alpha_3$ on the binding of entactin-coated beads to immune complex-stimulated PMN. These data indicate that the binding of entactin to $\alpha_3\beta_1$ on immune complex-stimulated PMN does not require the RGD sequence within the entactin molecule.

Ligation of $\alpha_3\beta_1$ by the G2 Domain of Entactin Stimulates Fc Receptor-mediated Phagocytosis—To ascertain which domains of the entactin molecule were responsible for binding $\alpha_3\beta_1$ and for signaling for enhanced ingestion, we examined the ability of fusion proteins encompassing the G1, G2, and E domains of entactin to stimulate ingestion of ElG via $\alpha_3\beta_1$ and to bind to stimulated PMN via $\alpha_3\beta_1$. The G2 domain fusion protein stimulated ingestion of ElG, and this stimulation was abrogated completely by inclusion of anti-$\alpha_3$ (Fig. 4A). GST alone had no effect on the level of ingestion (data not shown). Thus, ligation of $\alpha_3\beta_1$ by the G2 domain is sufficient to signal for augmentation of Fc receptor-mediated ingestion. To ascertain if the G2 domain fusion protein could also bind to PMN via $\alpha_3\beta_1$, we assessed the binding of G2-coated beads to PMN either in the presence or absence of 30 nM PDBu as the stimulant. The G2-coated beads did not bind specifically to PMN in the absence of stimulation (Fig. 4B). However, PDBu stimulation markedly increased the binding of the G2-coated beads, and anti-$\alpha_3$ and $\beta_1$, but not anti-LRI, completely inhibited this specific binding (Fig. 4B). These data indicate that the G2 domain of entactin can bind specifically to $\alpha_3\beta_1$ expressed by PMN only after PMN activation by stimuli such as immune complexes or phorbol esters.

These data suggested that entactin contained at least two separate sites that activated two distinct PMN responses, chemotaxis mediated by the RGD sequence in the E domain and phagocytosis enhancement mediated by the G2 domain. Moreover, the second site was only revealed following stimulation of the PMN with either immune complexes or phorbol esters. To determine if stimulation of these different PMN functions was mutually exclusive and required no other regions of the entactin molecule, we compared the abilities of the isolated G2 and E domains to stimulate ingestion and to promote chemotaxis. As expected, the isolated G2 domain was fully able to augment Fc receptor-mediated ingestion (Fig. 5A), whereas it was completely unable to promote PMN chemotaxis (Fig. 5B). These data are consistent with the fact that the G2 domain does not bind specifically to unstimulated PMN and, therefore, would not be able to stimulate chemotactic activity (Fig. 4B). In contrast, the isolated E domain was fully able to promote PMN chemotaxis (Fig. 5B) but was insufficient for augmentation of ingestion (Fig. 5A). The inability of the E domain to stimulate ingestion could have been explained by a failure of the isolated domain to bind to stimulated PMN. To examine this, we assessed E domain-coated bead binding to PMN in the presence and absence of PDBu. E domain-coated beads bound specifically to both unstimulated and stimulated PMN- and anti-LRI-, but not anti-$\beta_1$-, inhibited binding (Fig. 6A). These data indicate that the isolated E domain can bind to both stimulated and unstimulated PMN but that ligation of LRI by the E domain is not sufficient to signal for augmentation of ingestion. These data demonstrate that the ability of entactin to promote these two physiologic separate functions resides within completely separate and non-overlapping domains.

As an additional control to show that these two functions resided uniquely within these domains, we examined the ability of the isolated G1 domain to augment ingestion and to promote chemotactic activity. It had no significant effect in either assay (data not shown). In addition, we examined the binding of G1 domain-coated beads to unstimulated and stimulated PMN and assessed the ability of anti-LRI and $\beta_1$ to inhibit binding. G1 domain-coated beads did bind specifically to PMN, and the binding was enhanced with PDBu stimulation.
Role of $\alpha_b\beta_3$ (CR3) in G2 Domain Binding and Stimulation of Phagocytosis—The $\beta_3$ integrins, and particularly CR3, play an important role in normal PMN function (4, 18, 22, 28). In addition to its well described recognition of specific ligands, including C3bi, fibrinogen, and ICAM-1, CR3 plays a more global role in PMN function, even when clearly described ligands for CR3 are not involved (18, 22). This includes Fe receptor-mediated phagocytosis (18) and may relate to interactions of CR3 with elements of the cytoskeleton (22, 29, 30). We have shown previously that PMNs from patients with leukocyte adhesion deficiency, which lack expression of $\beta_2$ integrins, fail to augment ingestion of IgG in response to numerous stimuli, including phorbol esters and matrix proteins (18). In addition, treatment of normal PMN with antibodies against either $\alpha_M$ or $\beta_2$ has the same effect (18). Therefore, we would predict that PMN treated with inhibitory antibodies against CR3 would not augment ingestion in response to the G2 domain of entactin. As expected, mAb LPM19C, which recognizes the I domain within $\alpha_M$ (31), inhibited G2 domain enhancement of IgG ingestion and binding of G2 domain-coated beads to PDBu-stimulated PMN (data not shown). In addition, LPM19C inhibited PDBu-stimulated ingestion of IgG (data not shown). While these data implicate CR3 in these functions, they do not differentiate between ligand binding of the G2 domain by CR3 or a more global role for CR3, such as reorganization of the cytoskeleton that could be required for enhanced phagocytosis. To discriminate between these possibilities, we performed the following experiments. First, we asked whether $\alpha_b\beta_3$ in the absence of CR3 would be capable of binding the G2 domain of entactin. We examined G2 domain-coated bead binding to NCI-H69 epithelial cells, which express primarily $\alpha_b\beta_1$ and do not express $\beta_2$ (23). G2 domain-coated beads bound specifically to these cells and their binding was inhibited by anti-$\beta_1$ and $\alpha_1$ (Fig. 6B). These data confirm the observation of Wu et al. (15) that $\alpha_b\beta_1$ transfected into CHO cells mediates adhesion to the G2 domain (15). Second, we asked whether CR3 expressed in the absence of $\alpha_b\beta_1$ would be capable of binding the G2 domain. We examined the binding of G2 domain-coated beads to PDBu-stimulated K562 erythroleukemia cells transfected with either RSV vectors encoding $\alpha_M\beta_2$ or RSV alone as a control. K562 cells express only $\alpha_M\beta_1$ constitutively (25) and do not express either LRI or $\alpha_b\beta_3$. As controls, we also examined the binding of fibronectin- and fibrinogen-coated beads. G2 domain-coated beads did not bind specifically to either the control (Fig. 7A) or the $\alpha_M\beta_2$-transfected cells (Fig. 7B). In contrast, fibrinogen-coated beads bound specifically to only the $\alpha_M\beta_2$-transfected cells, and this binding was decreased by mAb LPM19C (anti-$\alpha_M$) but not by an isotype control antibody to the level observed for HSA-bead binding (Fig. 7B). Assessment of C3bi binding gave similar results to those observed for fibrinogen-coated beads (data not shown). Specific fibronectin-bead binding was equivalent for both cell types, consistent with the constitutive expression of $\alpha_M\beta_1$ (Fig. 7, A and B). These data indicate that the G2 domain does not bind to $\alpha_M\beta_2$ under conditions that promote the binding of fibrinogen to $\alpha_M\beta_2$. Moreover, expression of $\alpha_b\beta_3$ in the absence of CR3 is sufficient for binding the G2 domain. Therefore, G2 domain binding to stimulated PMN is unlikely to involve direct binding of the G2 domain to $\alpha_M\beta_2$.
activities as full-length entactin. Matrilysin-digested entactin retained its capacity to stimulate ingestion by PMN via αβ1 (Fig. 8A) and to stimulate PMN chemotaxis (Fig. 8B). As a control to show that this activity was not the result of a non-specific effect of matrilysin cleavage on PMN function, we also digested RGE-entactin with matrilysin. RGE-entactin had no ability to promote PMN chemotaxis even when it had been digested with matrilysin (Fig. 8B). These data indicate that cleavage of entactin by an enzyme involved in matrix remodeling, which could occur at sites of inflammation, generated peptides that retained important PMN-stimulating activities.

**DISCUSSION**

We show in this work that multiple domains of entactin have the ability to ligate individual integrins expressed by PMN and to activate distinct PMN functions. Entactin contains at least two separate sites that activate different PMN functions. Entactin contains at least two separate sites that activate two distinct PMN responses, chemotaxis mediated by E domain ligation of LRI and phagocytosis mediated by G2 domain ligation of αβ1. The results of three separate experimental approaches were used to reach this conclusion. 1) Examination of GST-fusion proteins comprising the four separate domains of entactin for stimulation of PMN function and ligand binding indicated that the E and G2 domains stimulated different PMN functions and bound two separate integrins; 2) cleavage of entactin by matrilysin liberated peptides that retained these separate PMN activating abilities; and 3) activation of PMN by immune complexes revealed a second ligand binding site within entactin and its separate function, thus spatially separating these two ligand-receptor interactions.

Entactin, like other extracellular matrix proteins including fibronectin, laminin, perlecan, and tenasin, is structurally organized into domains that mediate its disparate functions, such as matrix assembly, cell adhesion, and cell activation. For entactin, as well as other matrix molecules, the use of recombinant proteins that comprise these separate domains has allowed for definitive assignment of specific functions to specific domains. For example, the use of fusion proteins has allowed the assignment of the network forming, heparin binding, and αβ1 integrin binding sites of laminin-1 to the α-chain of its short arm (34). Similarly, the G2 domain of entactin has been definitively assigned as the binding site for the 29-kDa aminoterminal fragment of fibronectin (21), thus extending the biologic role of this domain in matrix assembly because it also binds type IV collagen (12) and the core protein of heparan sulfate proteoglycan (35). In addition to its role in matrix assembly, the G2 domain is also a major cell attachment site and promotes adhesion of mouse mammary tumor cells (14), human melanoma cells (14), and CHO cells transfected with αβ1 (15). This cell adhesion site has been further mapped to amino acid residues 358–396 and is included within the first EGF homology repeat of entactin located at the carboxyl-terminal end of the thread-like connection linking G1 and G2. Thus the cell attachment site is not located within the globular domain of G2 but is in the EGF repeat flanking its amino terminus (14). Furthermore, the other cell attachment site in entactin maps to the first EGF repeat within the E domain and contains the single RGD sequence (14). These data suggest that the EGF homology repeats contain the appropriate framework for presentation of adhesion recognition motifs to specific integrin re-
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13. Durkin, M., Chakravarti, S., Bartos, B., Liu, S.-H., Friedman, R., and Chung, A. (1990) J. Biol. Chem. 265, 2511–2526
14. Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H., Fox, J., Mayer, U., Nischt, R., Aumailley, M., Reinhardt, D., Wiedemann, H.
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Lewis, M., and Chung, A. E. (1990) *J. Biol. Chem.* **265**, 5188–5191

Sires, U. I., Griffin, G. L., Broekelmann, T. J., Mecham, R. P., Murphy, G., Chung, A. E., Welgus, H. G., and Senior, R. M. (1993) *J. Biol. Chem.* **268**, 2069–2974

Haiech, J.-C., Wu, C., and Chung, A. (1994) *Biochem. Biophys. Res. Commun.* **199**, 1509–1517

Graham, I., Anderson, D. C., Holers, V., and Brown, E. (1994) *J. Cell Biol.* **127**, 1139–1147

Elices, M. J., Urry, L. A., and Hemler, M. E. (1991) *J. Cell Biol.* **112**, 169–181

Dedhar, S., Jewell, K., Rojiani, M., and Gray, V. (1992) *J. Biol. Chem.* **267**, 18908–18914

Pauß, R., Kovach, N., Harlan, J., and Ginsberg, M. (1993) *J. Cell Biol.* **121**, 155–162

Wayner, E., Gil, S., Murphy, G., Wilke, M., and Carter, W. (1993) *J. Cell Biol.* **121**, 1141–1152

Arroyo, A., Sanchez-Mateos, P., Campanero, R., Martin-Padura, I., Dejana, E., Sanchez-Madrid, R. (1992) *J. Cell Biol.* **117**, 659–670

Anderson, D. C., and Springer, T. A. (1991) *Annu. Rev. Med.* **38**, 175–194

Sharma, G. P., Exzel, R. M., and Arnaout, M. A. (1991) *J. Immunol.* **154**, 3461–3470

Fuortes, M., Jin, W., and Nathan, C. (1994) *J. Cell Biol.* **127**, 1477–1483

Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., Corbi, A. L., and Springer, T. A. (1992) *J. Cell Biol.* **120**, 1031–1043

Birkedal-Hansen, H., Moore, W. G., Boddien, M. K., Birkedal-Hansen, B., De Carlo, A., and Engler, J. A. (1993) *Crit. Rev. Oral Biol. Med.* **4**, 197–250

Murphy, G., and Doherty, A. J. P. (1992) *Am. J. Respir. Cell Mol. Biol.* **7**, 120–125

Colognato-Pyke, H., O’Rear, J. J., Yamada, Y., Carbonette, S., Cheng, Y.-S, and Yurchenco, P. D. (1995) *J. Biol. Chem.* **270**, 9398–9406

Battaglia, C., Mayer, U., Aumailley, M., and Timpl, R. (1992) *Eur. J. Biochem.* **206**, 359–366

Delwel, G. O., de Melker, A. A., Hugger, F., Jaspers, L. H., Flies, D. L., Kuijkman, I., Lindbloom, A., Paulsson, M., Timpl, R., and Sonnenberg, A. (1994) *Mol. Biol. Cell* **5**, 203–215

Vuori, K., and Ruoslahti, E. (1993) *J. Biol. Chem.* **268**, 21459–21462

Hendey, B., Klee, C. B., and Maxfield, F. R. (1992) *Science* **258**, 296–299

Ravetch, J. V. (1994) *Cell* **78**, 553–560

Van Strijp, J. A. G., Russell, D. G., Tuomanen, E., Brown, E. J., and Wright, S. D. (1993) *J. Immunol.* **151**, 3324–3336

Ishibashi, Y., Claus, S., and Relman, D. A. (1994) *J. Exp. Med.* **180**, 1225–1233