ENTAMOEBA HISTOLYTICA CYSTEINE PROTEINASE 5 BINDS INTEGRIN ON COLONIC CELLS AND STIMULATES NFκB-MEDIATED PRO-INFLAMMATORY RESPONSES

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Running Title: E. histolytica binds integrin

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Integrins are important mammalian receptors involved in normal cellular functions and the pathogenesis of inflammation and disease. Entamoeba histolytica is a protozoan parasite that colonizes the gut and in 10% of infected individuals causes amebic colitis and liver abscess resulting in 10⁶ deaths/year. E. histolytica-induced host inflammatory responses are critical in the pathogenesis of the disease, yet the host and parasite factors involved in disease are poorly defined. Here we show that pro-mature cysteine proteinase 5 (PCP5), a major virulent factor that is abundantly secreted and/or present on the surface of ameba binds via its RGD motif to αvβ3 integrin on Caco-2 colonic cells and stimulates NFκB-mediated pro-inflammatory responses. PCP5 RGD binding to αvβ3 integrin triggered ILK-mediated phosphorylation of Akt-473 that bound and induced the ubiquitination of NEMO. As NEMO is required for activation of the IKKa/IKKβ complex and NFκB signaling, these events markedly up-regulated pro-inflammatory mediator expressions in vitro in Caco-2 cells and in vivo in colonic loop studies in wild type and Muc2−/− mice lacking an intact protective mucus barrier. These results have revealed that EhPCP5 RGD motif is a ligand for αvβ3 integrin-mediated adhesion on colonic cells and represents a novel mechanism that E. histolytica trophozoites use to trigger an inflammatory response in the pathogenesis of intestinal amebiasis.

The colonic protozoan parasite Entamoeba histolytica (Eh) infects 10% of the world’s population and causes 100,000 deaths per year (1). Intestinal amebiasis is characterized by severe colitis and bloody dysentery. Unfortunately, we know little about the host and parasite factors involved in the sequence of events leading to the pathogenesis of intestinal amebiasis. Most evidence to date suggests a host response to E. histolytica components (e.g., Gal/GalNAc adherence lectin) following adherence to the epithelium or to inflammatory cells triggers an acute inflammatory response as an initiating factor central to the genesis of disease. Among the potential virulent factors that can play a role in disease are the parasite cysteine proteinases (CP). Cysteine proteinases are major virulent molecules produced by ameba of which EhCP1, EhCP2 and EhCP5 account for approximately 90% of all EhCP transcripts (2). Of these, EhCP5 is the major protease that is secreted and/or...
present on the ameba surface (3). EhCP5 is directly involved in disrupting the protective mucin barrier of the colon and in tissue invasion through degradation of extra-cellular matrix proteins, and plays a key role in immune evasion by degrading host antibodies and complement (4-6).

Integrins are αβ heterodimeric trans-membrane proteins located on the surface of mammalian cells that convey an intracellular signaling network controlling cellular processes such as cell-cell adhesion, differentiation, migration and survival (7-9). Integrins on the cell surface can also act as cellular receptors for soluble plasma proteins and a variety of microbial pathogens such as Borrelia burgdorferi, Yersinia spp., Bordetella pertussis, adenovirus and rotavirus (reviewed in 10). Pathogen recognition is mediated by the 3 amino acid RGD sequence arginine-glycine-aspartate (11). The expression of integrin is cell-type dependent and those that are expressed on the epithelium include several αβ integrins, which are present on the apical and around the lateral surfaces (8, 12). As proteases can interfere with cell adhesion and/or migration in a non-proteolytic fashion by integrin receptors (13-15), we explored whether EhCP5 can interact with colonic epithelial cell integrin to trigger host inflammatory responses, which are critical in the pathogenesis of intestinal amebiasis.

**Experimental Procedures**

**Cell lines, ameba components and reagents-** Caco-2 human colonic cells were obtained from the ATCC (Manassas, VA). Secreted proteinases (SP) were prepared as described previously (4). E. histolytica cultures deficient in CP5 was a gift from Dr. David Mirelman (Weizmann Institute of Science, Israel). NEMO (sc-8330), ILK, αβ3, β3, Ubiquitin (P4D1), αβ3 antibody, ILK siRNA (sc-35666, Santa Cruz), ILK shRNA (RHS4430-101029656, Open Biosystem), human fibronectin, E64, PD98029, LY294002 and Wortmannin were obtained from Sigma. αβ3 blocking antibody LM609 (16) and αβ3 integrin protein were from Millipore; p-Akt, p-IκBα, His antibody and GSK3β fusion protein were from Cell signaling. GRGDSP and GRADSP peptide were purchased from Calbiochem. Expression of recombinant EhCP5- EhCP5 was expressed in E. coli strain BL21(DE3) [pPlacIQ] using the expression vector pJC45. Recombinant RAD and RGA were mutated by QuickChange site-directed mutagenesis kit (Stratagene). The recombinant protein was expressed as an insoluble histidine-tagged pro enzyme and was solubilized, purified, and refolded as described elsewhere (17). Protein purity was > 95% as revealed by SDS-PAGE.

**Mice colonic loop studies-** C57BL/6 wild type mice were purchased from Charles River and Muc2−/− of the same background were obtained from Dr. Anna Velcich (Montefiore Medical Center) and bred at the University of Calgary Animal Facilities. Mice were anesthetized with ketamine/xylazine, and colonic loops were injected with log-phase virulent wild-type trophozoites (10^6), PCP5 or PCP5-RAD (0.7 μg/μl). Sham-challenged mice were injected with 200 μl of the vehicle PBS and animals were kept on a 37°C warm plate for 3h. The colons were excised, and pro-inflammatory gene expression was analyzed by using quantitative Real Time PCR. All studies were carried out with the approval of the University of Calgary Animal Care Committee.

**Immunoprecipitation and Western blotting assay-** After treatment with various additions,
cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer [20 mM Tris-HCl pH 7.6, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 1mM Na3VO4, protease inhibitor cocktail (Sigma)]. Lysates were immunoprecipitated for 4h at 4°C with the appropriate primary antibody and protein A/G (Santa Cruz) was added overnight. Western blot analysis was performed as described previously (18). In vitro binding of purified αvβ3 (10nM) integrin protein to PCP5 (1µM) and mutants (1µM) lacking the RGD motif was also assessed by co-immunoprecipitation as described previously (13).

Immunofluorescence studies- Cells were fixed for 15 min with 3.7% paraformaldehyde, incubated with primary antibodies and subsequently with secondary antibodies. After the final washing, cells were covered with Permafluor mounting medium. Immunostained cells were viewed in a confocal microscope (OLYMPUS FV 1000 Confocal).

NFκB reporter assay- Caco-2 cells were co-transfected with NFκB-luc plasmid and pTK-Renilla luciferase plasmid as an internal control (ratio: 1:100) by using Lipofectamine™ 2000 (Invitrogen); 24h after transfection cells were treated as indicated. Cell lysates were measured using a Dual Luciferase Reporter Assay system (Promega) and all data were normalized using pTK-Renilla luciferase. Results are expressed as fold increase over matched controls.

Cell attachment assay- Cell attachment assay was performed as described previously (13). Briefly, 96-microwell plates were coated with PCP5, RGD, RAD, fibronectin and BSA as a control (all at 5ug/ml) in 100µl PBS/well for 18h at 4°C. The wells were blocked with 10 mg/ml BSA for 30 min. Sub-confluent Caco-2 were detached using trypsin/EDTA, and re-suspended at 2×10^5 cells/ml in DMEM/F12 containing 1% BSA, and 100µl of cells were added to each well. Inhibition studies were performed by pre-incubating Caco-2 cells with αvβ3 antibody (5µg/ml), GRGDSP (50µg/ml), and GRADSP (50µg/ml) for 30 min before addition to the wells. Control cells were treated with BSA (5µg/ml). The plates were incubated for 90 min at 37 °C in 5% CO2. Cells were washed with PBS, fixed with 4% formaldehyde in PBS, stained with 0.1% crystal violet for 20 min, and washed, dissolved, and cell numbers were quantified by measurement of the optical density at 595 nm in a microplate reader.

Quantitative Real Time PCR- Total RNA from colon was isolated using RNeasy Mini kit (QIAGEN) and analyzed by Real Time PCR with SyBr Green (QIAGE). mRNA expression was normalized against GAPDH. Fold change over control was determined according to the Ct method (19).

In vitro ILK assay- ILK assay was described previously (20). Caco-2 cells were treated as indicate. Cells were lysed and immunoprecipitated with 4 µg of anti-ILK antibody. 1µg of GSK3β fusion protein (Cell Signaling) was then added to the immunoprecipitated lysates buffer followed by incubation for 30 min at 30 °C. After reaction, 20 µl of SDS sample buffer was added to terminate reaction. Samples were analyzed by Western analysis with phospho-GSK3β and total GSK3β antibodies.

Statistical analysis- The values for each parameter within a group are expressed as the mean ± SEM. Statistical significance was determined by Student’s t-test (when comparing two groups) or analysis of variance with a post hoc test (when comparing more than two groups).
Significance was defined as $P<0.05$.

**RESULTS**

*E. histolytica* PCP5 interacts with integrin and activates NF$\kappa$B. We have previously shown (4) that the specific cysteine protease inhibitor E-64 markedly inhibited mucin degradation by *E. histolytica*-secreted proteinases (SP). To determine whether ameba SP can directly activate colonic cells we performed a time-dependent study to quantify the activation of NF$\kappa$B. As shown in Fig. 1A, ameba SP rapidly induced the ubiquitination of NEMO and the phosphorylation of I$\kappa$B$\alpha$ (Fig. 1B) even in the presence of the cysteine proteinase inhibitor E64, suggesting that cysteine proteinase enzyme activity was not required for activating NF$\kappa$B. Phosphorylation of I$\kappa$B$\alpha$ is catalyzed by I$\kappa$B kinase (IKK), a complex composed of three subunits, IKKa/IKK1, IKK$\beta$/IKK2, and NEMO/IKK$\gamma$. IKK1 and IKK2 are the catalytic subunits, whereas NEMO serves a non-enzymatic, regulatory function (21, 22). IKK$a$ and IKK$\beta$ are serine/threonine protein kinases, whereas NEMO contains several protein interaction motifs but no apparent catalytic domains (23). Genetic studies suggest that NEMO is absolutely required for the activation of IKK and NF$\kappa$B in response to diverse stimuli (24, 25) and ubiquitination of NEMO is required for the activation IKK (26). *E. histolytica* intrinsic and secreted proteinases contain both pro-mature CP5 (PCP5) and mature CP5 (27). Interestingly, when ameba was grown in the presence of E64, PCP5 levels were significantly increased (Fig. 1C) as compared to untreated controls. This finding is consistent with a previous report that showed ameba secretes both PCP5 and mature CP5 (27). Thus, to elucidate a specific functional role for EhCP5, colonic cells were exposed to secreted proteinases derived from wild-type (WT), amebapore A vector control (AP$^+$) or *E. histolytica* genetically silenced for CP5 (AP/CPCP$^+$) expression. Under these conditions, the WT or vector control (AP$^+$) significantly induced the ubiquitination of NEMO, but CP5$^-$ did not (Fig. 1D). These data clearly show that PCP5 was the critical proteinase involved in activating NF$\kappa$B and inducing the ubiquitination of NEMO.

As inhibition of CP activity did not prevent CP-induced ubiquitination of NEMO and phosphorylation of I$\kappa$B, we were led to inquire whether CP5 was directly binding and interacting with an epithelial cell surface receptor to activate NF$\kappa$B. Amino acid sequence analysis revealed that PCP5 encodes an RGD motif (92-94 aa, Fig. 2A), the peptide motif recognized by integrin receptors, in the prodomain region upstream toward the carboxyl terminal end. The RGD motif is also found on EhCP18 and EhCP112 within the catalytic domain; however these enzymes are not highly expressed or secreted by ameba (3). Based on this finding, we postulated that PCP5 might function as a ligand with integrin to induce pro-inflammatory responses. $\alpha_v\beta_3$ integrin is abundantly expressed on Caco-2 cells in culture and colonic epithelial cells and binds RGD motifs (28). Thus, to determine binding of PCP5 to $\alpha_v\beta_3$, co-immunoprecipitation studies were done which demonstrated that PCP5 bound $\alpha_v\beta_3$ integrin on Caco-2 cells (Fig. 2B). However, a single amino acid substitution in the RGD motif where either the aspartate or glycine residue was substituted by an alanine residue to generate the mutants RAD or RGA, respectively (Fig. 2A), abrogated binding (Fig. 2B). Similarly, Caco-2 cells...
were pretreated with the GRGDSP peptide that specifically binds integrin (but not to the non-specific GRADSP peptide), as well as neutralizing antibodies against $\alpha_\nu\beta_3$ markedly inhibited PCP5 binding to $\alpha_\nu\beta_3$ integrin. Moreover co-immunoprecipitation of purified recombinant PCP5 alone-demonstrated PCP5 bound to the $\alpha_\nu\beta_3$ receptor whereas the mutants PCP5 RAD and RGA did not (Fig. 2C). As integrin $\alpha_\nu\beta_3$ is also expressed on the apical surface of many human cells types including colonic epithelial cells (12), we determined the binding of PCP5 to Caco-2 monolayer. As shown in Fig. 2D, cell attachment analysis revealed that PCP5 bound $\alpha_\nu\beta_3$ integrin whereas the mutants RAD and RGD did not, However, cells pretreated with GRGDSP and $\alpha_\nu\beta_3$ antibody inhibited PCP5 binding to $\alpha_\nu\beta_3$ integrin. To determine cellular localization of the $\alpha_\nu\beta_3$ integrin, Caco-2 cells were exposed with PCP5 and analyzed by confocal fluorescence microscopy. As shown in the merged photomicrograph (Fig. 2E, arrows), PCP5 co-localized with $\alpha_\nu\beta_3$ on the surface of the colonic cells. Taken together, these findings demonstrate that PCP5 bound to $\alpha_\nu\beta_3$ integrin receptor on colonic cells via the RGD motif.

\textit{E. histolytica} PCP5 activates NF\kappa B and phosphorylates Akt. Integrin linked kinase (ILK) is implicated in the direct phosphorylation of serine 473 of Akt (29). ILK functions downstream of PI3-kinase and promotes the phosphorylation of serine 473 of Akt (30). We have recently shown that \textit{E. histolytica} can activate the PI3 kinase/Akt pathway leading to NF\kappa B-mediated pro-inflammatory MCP-1 expression and secretion by inducing the phosphorylation of Akt (31). Here we show that PCP5 can induce the phosphorylation of Akt-473 rather than Akt-308 in a dose- and time-dependent manner (Fig. S1A and B). In particular, PCP5 significantly induced the phosphorylation of Akt-473 whereas the PCP5 mutants RAD and RGA, had no effect (Fig. 3A), clearly implicating that the RGD motif is required and essential for PCP5-induced Akt phosphorylation. As predicted, compared to the control peptide GRADSP, GRGDSP significantly inhibited PCP5-mediated phosphorylation of Akt (Fig. 3B). Moreover, the PI3K inhibitors Ly294002 (LY) and Wortmannin (WM) significantly abolished PCP5-mediated phosphorylation of Akt (Fig. 3C). As activated ILK can phosphorylate the substrate GSK3\beta \textit{in vitro} (20), our results show PCP5-induced ILK activation whereas the mutants RAD and RGD did not. Moreover, the peptide GRGDSP and neutralizing antibodies against $\alpha_\nu\beta_3$ inhibited ILK activation in response to PCP5 (Fig. S1C).

It is well known that ligand coupling through the $\alpha_\nu\beta_3$ integrin dependently induces the activation of NF\kappa B (32). Although PCP5 can significantly induce NF\kappa B activity (Fig. S2A and B, Fig. 3D), the RAD and RGA mutants did not (Fig. S3A). As the GRGDSP peptide can also inhibit PCP5-induced phosphorylation of I\kappa B\alpha (Fig. 3E, Fig. S3B), this suggests that the RGD motif of PCP5 is essential for PCP5-induced NF\kappa B activity. In support of this, inhibiting the phosphorylation of Akt with LY and WM markedly decreased PCP5-induced phosphorylation of I\kappa B\alpha (Fig. 3F, Fig. S3C). These results were corroborated by NF\kappa B luciferase activity analysis (Fig. 3G and H) where PCP5 (or PCP5 treated with polymycin B to remove any potential LPS contaminant) stimulated high NF\kappa B activity. Fibronectin, as a non-specific ligand for $\alpha_\nu\beta_3$ integrin (8, 33) also induced robust NF\kappa B activation in
Caco-2 cells (Fig. S4B). Considering the non-specific effects of siRNA on ILK (34), we silenced ILK using siRNA or shRNA. As shown in Fig. S4A, silencing ILK reduced PCP5 ILK-mediated phosphorylation of IkBa and the phosphorylation Akt. These findings clearly show that PCP5 binding to α,β3 led to the activation of the ILK/Akt/NFκB pathways. 

**PCP5 mediates the Akt/NEMO pathway.** Ubiquitination of NEMO is required for NFκB activation (24-26). The Akt/IKK pathway mediates cell survival (35, 36) and pro-inflammatory responses (31). Here we found that PCP5 but not the mutants RAD and RGA can rapidly induce the ubiquitination of NEMO (Fig. 4A and B, Fig. S5). Akt induces NFκB activity by activating IKK (35, 36), and as expected, we show that inhibitors of Akt (LY and WM) abrogated PCP5-induced ubiquitination of NEMO (Fig. 4C). As shown in Fig. 4D, PCP5-induced Akt activation resulted in Akt binding to NEMO, while the mutants, RAD and RGA and PCP5 with addition of the competitor peptide, GRGDSP did not. Under these conditions, the PI3K inhibitors LY and WM completely inhibited PCP5-induced interaction between Akt and NEMO (Fig. 4E). As ILK functions downstream of PI3 kinase and promotes the phosphorylation of Akt (29, 30) as predicted, silencing ILK by siRNA or shRNA abolished PCP5-induced ubiquitination of NEMO, as well as Akt binding to NEMO (Fig. 4F and G). Taken together, these findings clearly implicate PCP5 binding to α,β3 integrin, which in turn triggers ILK/Akt/NEMO signaling in colonic epithelial cells.

**PCP5 induces pro-inflammatory responses in vitro and in vivo.** As ameba SP can induce Akt and NFκB-mediated pro-inflammatory responses (31), we determined if PCP5 coupling through integrin could activate the Akt/NFκB pathway resulting in pro-inflammatory responses. As shown in Fig. 5A and B, PCP5-induced the secretion of the pro-inflammatory cytokines TNF-α and IL-1β by Caco-2 cells; however, cells pretreated with α,β3 antibody, GRGDSP or Akt inhibitor, abolished PCP5-induced pro-inflammatory responses. To determine if a similar response occurred in vivo, we used an animal model of the disease and demonstrated that wild type mice colons infected with live highly virulent *E. histolytica* trophozoites or challenged with purified PCP5 stimulated robust TNF-α, IL-1β, IL-6 and Cox-2 gene expression (Fig. 6A-D). In parallel, mice infected with CP5− ameba or inoculated with the RAD-PCP5 mutant, could not mimic the effect of virulent *E. histolytica* or PCP5 (Fig. 6A-D). As ameba CPs have high enzyme activity against Muc2 mucin substrates (37), which can substantially affect PCP5 binding to α,β3 integrin receptor on colonic cells, studies were done in Muc2− mice, which are deficient for the major structural component of colonic mucus, to facilitate direct binding of PCP5 to the surface of the epithelium. Surprisingly, in Muc2− mice pro-inflammatory cytokine gene expressions were significantly up regulated in response to PCP5 but not to the mutant RAD compared to WT mice with an intact mucus barrier (Fig 6. E-H). These results suggest that loss of Muc2 mucosal barrier protective function led to enhance interaction of PCP5 with the integrin receptor on colonic cells (Fig. 5). Moreover, Muc2− mice colons pretreated with blocking α,β3 integrin antibody significantly inhibited *E. histolytica*-induced pro-inflammatory gene expression (Fig. S5A-D), suggesting that the α,β3 integrin pathway is critically involved in *E. histolytica*-mediated...
DISCUSSION

The pathogenesis of intestinal amebiasis is a multifaceted event involving both host and parasite factors. Host inflammatory responses are critical in the pathogenesis of intestinal amebiasis (38). In this study, we have revealed a novel role for integrins in the pathogenesis of *E. histolytica*, making $\alpha_\nu\beta_3$ integrin the first human receptor to be exploited by a colonic mucosal parasite. Our findings support an intriguing mechanism by which the PCP5 RGD motif can bind $\alpha_\nu\beta_3$ integrin to trigger integrin/NF$\kappa$B signaling that elicits pro-inflammatory cytokine production.

*E. histolytica* cysteine proteinases are key virulence factors involved in pathogenesis (3, 4). Trophozoites secrete both pro-mature CP5 and mature CP5 (27). CP5-deficient trophozoites are known to inflict less gut inflammation and damage to the intestinal barrier and are incapable of inducing amebic liver abscesses (39). Thus, it was not surprising to find that *E. histolytica* genetically deficient in CP5 production elicited significantly less NF$\kappa$B activation and pro-inflammatory responses; reinforcing the notion that CP5 is an important virulence factor. Importantly, as both pro-mature CP5 and mature CP5 are secreted, it was critical to determine which form of the enzyme was essential for activating NF$\kappa$B-mediated pro-inflammatory responses. With the use of the specific CP5 enzyme inhibitor E64 to abolish CP5 enzyme activity, we were intrigued that it had no effect on PCP5-mediated NF$\kappa$B activation, suggesting that pro-mature CP5 (PCP5) may activate NF$\kappa$B. Although previous studies have shown that inhibiting cysteine proteinase activity decreases *E. histolytica*-induced liver abscesses formation, it is important to note that only E64-resistant ameba cultures, rather than wild-type ameba cultured with E64, had a significant effect on liver abscesses formation (27). As E64-resistant *E. histolytica* cultures have significantly decreased CP5 gene expression (27), this finding demonstrates precedence of CP5 as a virulent factor for *E. histolytica*. Interestingly, our results show that the pro-mature form, PCP5, is also an important virulent factor and in this regard, it is the first report to ascribe a functional role for PCP5. These findings also explain why *E. histolytica* cultured with E64 cannot inhibit liver abscesses formation (27), as CP5 enzyme activity is not required for the NF$\kappa$B-mediated pro-inflammatory responses that potentiate amebic liver abscess formation (reviewed in 38).

Amino acid sequence analysis revealed that PCP5 encodes an RGD motif, which is known to interact with $\alpha_\nu\beta_3$ integrin to activate NF$\kappa$B (31). Other integrin including $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_1$ can recognize ligands containing an RGD tripeptide active site (8). Even though $\alpha_v\beta_3$ integrin are abundantly expressed on Caco-2 and colonic epithelial cells (28), we cannot exclude the possibility that PCP5 may bind other integrins and have a functional role. Our results show that the RGD motif was required for PCP5-mediated NF$\kappa$B activation by inducing ubiquitination of NEMO. NEMO is the regulatory subunit of the IKKa/IKKB complex, which predominantly acting through IKKB phosphorylates IxBs that sequester NF$\kappa$B in the cytoplasm. This phosphorylation event signals IxB ubiquitin-mediated degradation, and thereby the release of NF$\kappa$B into the nucleus where it activates pro-inflammatory gene expression. This pathway of NF$\kappa$B activation is dependent on NEMO as...
pro-inflammatory signal induced NfκB activity is completely blocked in NEMO-deficient cells but only partially affected in IKK2/β-deficient cells (40, 41). Integrin-linked kinase (ILK) has been implicated in direct phosphorylation of Akt serine 473 (29) and functions downstream of PI3-kinase in promoting phosphorylation of Akt serine 473 (30). We have previously shown that proteins secreted by virulent E. histolytica activate the PI3 kinase/Akt pathway leading to NfκB-mediated pro-inflammatory MCP-1 expression by inducing Akt phosphorylation at serine 308 (30). Here we found that PCP5 had no effect on the phosphorylation of Akt308. Unexpectedly, high concentrations of PCP5 (>500 µg/ml) decreased the phosphorylation of Akt473 and IκBα in a time-dependent fashion suggesting that PCP5 may function within a narrow range, but this is only speculation. Silencing ILK abolished ILK-mediated phosphorylation of Akt 473. These data suggest that PCP5 integrin binding activated the ILK/Akt pathway and activated NfκB. The Akt/IKK pathway is known to mediate cell survival (35, 36) and pro-inflammatory responses (31), however, it is still unclear how Akt induce the activation of NEMO and activate downstream NfκB signaling. Our results show that Akt bound to NEMO and caused NEMO to be ubiquitinated, and that NEMO ubiquitination in turn led to the activation of IKK and subsequent activation of NfκB. These results not only clarify the mechanism whereby PCP5 activates NfκB, but also provide a novel mechanism of Akt interaction with NEMO. Activation of the NfκB family mediates inflammation (42, 43). Thus, it was not surprising that PCP5 significantly induced pro-inflammatory cytokine secretion (TNF-α and IL-1β) in human colonic cells. More importantly, mice colons challenged with wild type virulent E. histolytica trophozoites and recombinant PCP5 induced robust pro-inflammatory cytokine gene expression (TNF-α, IL-1β, Cox-2, IL-6), whereas, CP5−/− deficient trophozoites and a RAD-PCP5 mutant elicited a greatly reduced pro-inflammatory response. We have previously shown that CP5 has high enzyme activity against MUC2 colonic mucin (37), which can substantially prevent CP5 from engaging epithelial cell integrin in the colon. Accordingly, studies done in Muc2−/− mice showed a significant increased in pro-inflammatory cytokine gene expression as compared to wild-type mice, clearly implicating that loss of the protective mucus barrier led to enhanced interaction of PCP5 with the epithelial integrin receptor. This study substantiates our hypothesis that secreted CP5/PCP5 can exert multiple functions in the gut lumen by first exhibiting enzyme activity against mucin, and only when the luminal barrier is compromised PCP5 binds integrin on colonic cells via the RGD motif. This step-wise mode of action for PCP5 in the pathogenesis of intestinal amebiasis seems the most likely scenario.

Taken together, initiation of host pro-inflammatory responses through PCP5 binding to αvβ3 integrin on colonic cells via the activation of the ILK/Akt/NEMO pathway provide novel insights into the molecular basis of E. histolytica-induced intestinal inflammation; the central event involved in instigating amebic colitis.

REFERENCES

Switzerland.

FOOTNOTES

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FIGURE LEGENDS

Fig 1. PCP5 is essential for NFκB activation. A, Caco-2 cells at 90% confluence were treated with SP (100µg/ml) for various times and SP + E64 (100µM) for 10 min. E64 at 100µM inhibited the enzymatic activity of SP and the degradation of the synthetic substrate Z-Arg-Arg-pNA by >95%. Cell lysates were subjected to immunoprecipitation by using
NEMO antibody, and NEMO ubiquitination was detected by using an ubiquitin antibody. Control cells were treated with PBS. B, Caco-2 cells were treated with SP (100µg/ml) for various times and SP + E64 for 10 min, respectively, p-IκBα was detected by Western blotting. C, *E. histolytica* trophozoites were treated with or without E64, and the supernatant collected and CP5 expression detected by Western blotting. NS: non-specific, CP: Cysteine proteinase. D, Fresh passage WT, *AP*−/− (vector control) and *AP*−/− *CP5*−/− deficient *E. histolytica* were exposed to Caco-2 cells for 10 min, respectively. Control cells were treated with fresh medium, washed with PBS and cell lysates were subjected to immunoprecipitation using NEMO antibody. The ubiquitination of NEMO was detected by an ubiquitin antibody.

**Fig. 2.** PCP5 RGD-dependent binding to the αvβ3 receptor. A, Schematic representation of the PCP5 constructs illustrating the pro-domain, the mature CP5 regions and the RGD sequence and the mutants. B, Caco-2 cells were treated with PCP5 (300µg/ml), RAD and RGA (300µg/ml) or pretreated with αvβ3 antibody (5µg/ml), GRGDSP (50µg/ml), GRADSP (50µg/ml), IgG (5µg/ml, controls) for 30 min, respectively, and then treated with PCP5 (300µg/ml) for 10 min. Cell lysates were subjected to immunoprecipitation by using an αvβ3 antibody. C, Direct binding of purified PCP5 to αvβ3 integrin. D, Caco-2 cells were pretreated with αvβ3 antibody (5µg/ml), GRGDSP (50µg/ml), GRADSP (50µg/ml) or IgG (5µg/ml, controls) for 30 min, respectively, and then treated with PCP5 (300µg/ml) for 10 min. PCP5 binding to αvβ3 was assayed by cell attachment method as described in experimental procedures. Data are triplicates from three independent experiments. Asterisk, P<0.05. Error bars indicate SEM. E, Confocal fluorescence microscopy of Caco-2 cells expressing αvβ3 or his-PCP5. Original magnification ×40. Arrows shows co-localization of PCP5 and αvβ3.

**Fig. 3.** PCP5/Akt induces NFκB activation. A, Caco-2 cells were treated with PCP5 (300µg/ml), RAD and RGA (300µg/ml) or (B) pretreatment with GRGDSP (50µg/ml), and GRADSP (50µg/ml), or (C) 20 nM Wortmannin and 10 µM LY294002 for 30 min, respectively, and then stimulated with PCP5 (300µg/ml) for 10 min. Akt phosphorylation was assayed by Western blotting. D-F, Caco-2 cells were treated as indicated above and p-IκBα was detected by Western blotting. G, NFκB transcriptional activity was assayed by dual luciferase analysis using the agonist concentrations listed above; PBS was used as a control. (H) Cells were pretreated with αvβ3 antibody (5µg/ml) or control IgG (5µg/ml) for 30min followed by treatment with PCP5 for 12h and cell lysates were assayed by luciferase analysis, Data are triplicates from three independent experiments.

**Fig. 4.** PCP5 induces the ubiquitination of NEMO by enhancing Akt binding to NEMO. A-C, Caco-2 cells were treated with PCP5, RAD and RGA or pretreated with GRGDSP, GRADSP, LY and WM for 30 min, respectively (same concentrations as in Fig. 3), and then treated with PCP5 for 10 mins, and immunoprecipitation analysis was performed as described in methods. Ubiquitination of NEMO was detected by Western blotting. D-E, Caco-2 cells were treated as above, and immunoprecipitation analysis was performed and Akt and NEMO were detected by Western blotting. F, Caco-2 cells were transfected with control siRNA or ILK siRNA for 48h, and then cells were treated with or without PCP5 (300µg/ml) for 10 min. Cell lysates were subjected to immunoprecipitation and the ubiquitination of NEMO was
detected by Western blotting. G, Caco-2 cells were transfected with ILK siRNA (as above) and immunoprecipitation analysis was performed.

**Fig. 5.** PCP5 stimulates pro-inflammatory cytokine production by Caco-2 cells. Cells were treated with PCP5, RAD, RGA, or pretreatment with αvβ3 (5µg/ml) antibody, GRGDSP (50µg/ml), GRADSP (50µg/ml) or IgG (5µg/ml, controls) for 30 min, respectively. A. TNF-α and B, IL-1β protein release were detected by ELISA after 8 h as described in experimental procedures. Asterisk, P<0.05. Error bars indicate SEM.

**Fig. 6.** PCP5 induces pro-inflammatory responses in mouse colon. **A-D.** Wild type (WT) and Muc2−/− mice colons were infected with virulent *E. histolytica* trophozoites and *AP−/−CP5−/−* deficient *E. histolytica* trophozoites for 3h as described in experimental procedures. TNF-α, IL-1β, IL-6, Cox-2 gene expression were detected by Real-Time PCR. Asterisk, P<0.01; Double asterisk, P<0.05. Error bars indicate SEM. **E-H,** WT and Muc2−/− mice colons were treated with PCP5 and PCP5-RAD for 3 h, and TNF-α, IL-1β, IL-6, Cox-2 gene expression were detected by Real-Time PCR. Asterisk, P<0.01; double asterisk, P<0.05. Error bars indicate SEM.
Figure 1

A

(KDa) 0 5 10 30 10 min
IP: NEMO 170 130 95 72 55
IB: UB

55 Lysate
NEMO

B

(KDa) 0 5 10 15 30 10 min
p-IkBα 43 43
Actin

C

(KDa) SP SP+E64 PCP5
Ub 34 26

PCP5 NS CP5

D

(KDa) C WT AP AP CP5
Ub 170 130 95 72 55

NEMO 55 Lysate
Figure 2

A

pro-mature CP5

ERMIN 90-94
RGD
Catalytic domain
pro-domain
mature CP5 (94-318)

B

(KDa)

IP: αβ3

34

34

130

PCP5

RAD

RGA

αβ3

PCP5

His

E

DAPI

αβ3

His

Merge

C

(KDa)

IP: αβ3

34

130

PCP5

RAD

RGA

His

β3

D

Net 595

PCP5 RAD RGA αβ3 GRGDS GRADSP FN
Figure 5

A

B