The Extra Domain A of Fibronectin Activates Toll-like Receptor 4*  

Received for publication, January 5, 2001  
Published, JBC Papers in Press, January 9, 2001, DOI 10.1074/jbc.M100999200

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Cellular fibronectin, which contains an alternatively spliced exon encoding type III repeat extra domain A (EDA), is produced in response to tissue injury. Fragments of fibronectin have been implicated in physiological and pathological processes, especially tissue remodeling associated with inflammation. Because EDA-containing fibronectin fragments produce cellular responses similar to those provoked by bacterial lipopolysaccharide (LPS), we examined the ability of recombinant EDA to activate Toll-like receptor 4 (TLR4), the signaling receptor stimulated by LPS. We found that recombinant EDA, but not other recombinant fibronectin domains, activates human TLR4 expressed in a cell type (HEK 293 cells) that normally lacks this Toll-like receptor. EDA stimulation of TLR4 was dependent upon co-expression of MD-2, a TLR4 accessory protein. Unlike LPS, the activity of EDA was heat-sensitive and persisted in the presence of the LPS-binding antibiotic polymyxin B and a potent LPS antagonist, E5564, which completely suppressed LPS activation of TLR4. These observations provided a mechanism by which EDA-containing fibronectin fragments promote expression of genes involved in the inflammatory response.

Because of the similarities between the effects of the EDA domain and LPS, we hypothesized that these molecules may be recognized by the same receptor.

Constituents of pathogens, including LPS, are detected by a family of recently discovered receptors related to the Drosophila Toll protein, which is involved in dorsoventral polarization and the induction of antimicrobial factors in response to infection (9, 10). The mammalian Toll-like receptors have an extra-cellular domain containing leucine-rich repeats and an intracellular domain, related to that of the IL-1 signaling receptor, that activates a signal transduction cascade resulting in nuclear translocation of nuclear factor kB (NF-kB) (9–15). The endogenous ligand for the Drosophila Toll receptor, spätzle, is a secreted protein that requires proteolytic activation (16, 17).

Mammalian TLR4 is the signaling receptor activated by LPS (9, 13–15, 18, 19), although the accessory proteins MD-2 (20) and CD14 (21) are required for maximal responses to LPS. The endogenous ligands/activators of the mammalian TLRs have not yet been identified, but it has been proposed that a proteolytic processing reaction generates the peptide ligands/activators in a manner analogous to the Drosophila Toll-spätzle system. Recent observations suggest that heat shock protein 60 (hsp 60) activates TLR4 (22), but it is not yet known if processing of hsp 60 is required to achieve TLR4 activation. In the present study, we determined that the EDA domain of fibronectin is capable of activating TLR4, which would account for the ability of EDA or EDA-containing fibronectin fragments to induce LPS-like responses.

**EXPERIMENTAL PROCEDURES**

Production of Recombinant Fibronectin Type III Repeat Proteins—Reverse transcription-polymerase chain reaction was employed to generate cDNAs encoding the individual fibronectin type III domains. The primers for EDA were: sense primer (EDA-s), 5′-CGGGATCCCAATGTGCCTCCCTAAAGG-3′; antisense primer (EDA-a), 5′-TCGGCCAGGGTGTACGCTCCAACT-3′; and antisense primer (EDA-a), 5′-TCGGCCAGGGTGTACGCTCCAACT-3′. The primers for EDB were: sense primer (EDB-s), 5′-CGGGATCCCAATGTGCCTCCCTAAAGG-3′; and antisense primer (EDB-a), 5′-TCGGCCAGGGTGTACGCTCCAACT-3′.

Reverse transcription-polymerase chain reaction amplification of hsp 60 is required to achieve TLR4 activation. In the present study, we determined that the EDA domain of fibronectin is capable of activating TLR4, which would account for the ability of EDA or EDA-containing fibronectin fragments to induce LPS-like responses.

* This work was supported by National Institutes of Health Grant HD-34612 and by a grant from the Bill and Melinda Gates Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: EDA, extra domain A; EDB, extra domain B; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; IL-1, interleukin 1; NF-kB, nuclear factor kB; III 1C, recombinant fibronectin type III repeat 1 C-terminus; III 11, fibronectin type III repeat 11; FBS, fetal bovine serum; MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbeco’s modified Eagle’s medium; sCD14, soluble CD14.
THP-1 Cell Experiments—THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml). Cells were resuspended in serum-free medium before each experiment and all studies were carried out in serum-free culture fluid. Cells were treated with recombinant fibronectin domains or LPS (serotype 055:B5) (Sigma) in the absence or presence of the LPS antagonist, E5531 (Eisai). The conditioned medium was collected for analysis of matrix metalloproteinase 9 (MMP-9) expression by zymography or ELISA. Cell numbers were counted with a hemocytometer.

Assay of MMP-9—Conditioned medium was subjected to zymography as previously described (22). MMP-9 released into the culture fluid was also measured using an MMP-9 ELISA kit (Oncogene Research Products, Boston, MA).

Western Blot Analysis—Cells were lysed in Nonidet P-40 lysis buffer, and nuclear extracts were prepared and protein quantitated using the Micro BCA protein assay (Pierce). Nuclear extracts (15 μg/lane) were subjected to Western blotting using antibodies raised against human NF-κB p65 and p50 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Activation of TLR4—For transfection experiments, HEK 293 cells, which do not normally express TLR4, were plated in 48-well tissue culture plates at a density of 5 × 105 cells/well and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS for 24 h. Cells were then transfected with TLR4 cDNA or empty vector and/or MD-2 cDNA plus 50 ng of pELAM-1-luc using a calcium phosphate protocol (13). All cells were also transfected with pRL-TK plasmid, a Renilla luciferase control reporter vector (Promega, Madison, WI) to normalize transfection efficiencies. After transfection, cells were maintained in DMEM supplemented with 10% FBS for 24 h. The medium was subsequently removed and replaced with DMEM plus 0.5% FBS. The cells were either left untreated or incubated with 1 μM of the indicated recombinant fibronectin domain or 100 ng/ml LPS plus 10 μg/ml soluble CD14 (sCD14) for 18 h. Cells were harvested in lysis buffer and assayed for Firefly and Renilla luciferase activity as described by the manufacturer of the Dual Luciferase Reporter System (Promega).

HEK 293 cells stably carrying plasmids for TLR4, MD2, and ELAM-1-luciferase (HEK-TLR4/MD2/ELAM-1-luc) were generated as described (15). Cells were plated in 96-well plates at a density of 50,000 cells/well and maintained in DMEM plus 10% FBS for 24 h. The medium was removed and replaced with DMEM plus 0.5% FBS and the cells were incubated with 1 μM of the indicated fibronectin domain protein or 10 or 100 ng/ml LPS plus sCD14 and, in some cases, in the presence of 1 μM E5564 or 10 μg/ml polymyxin B for 18 h. Fibronectin domain proteins and LPS were also heat-treated at 95 °C for 20–60 min prior to addition to the cells. Steady-Glo reagent (Promega) was added to the wells and the amount of luciferase activity was quantified. Data are shown as means ± S.D. from one representative experiment in which each transfection or treatment was performed in triplicate. Each experiment was repeated on at least three separate occasions.

Mouse Splenocyte Assay—Spleens from C3H/HeN (control; Charles River Laboratories) and eC3H/HeJ (LPS-insensitive because of Tlr4 mutation; Jackson Laboratories) mice were broken apart with sterile forceps and passed through a sterile 21-gauge needle. Splenocytes were collected, washed once in serum-free RPMI 1640 medium and then seeded into 24 well tissue culture plates at a density of 5 × 105 cells/well in 500 μl of RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml β-mercaptoethanol and 100 units/ml penicillin and streptomycin. Recombinant proteins (1 μM) or LPS (100 ng/ml) were added and the cells cultured for 72 h at 37 °C. Supernatants were collected and stored at −80 °C until assayed for IL-10 using a specific ELISA (Endogen, Inc.).

RESULTS AND DISCUSSION

We first characterized the activities of recombinant fibronectin type III repeat domains on cells that express TLR4, human THP-1 monocyte/macrophage cells, using production of MMP-9 as an index of the cellular response. Gelatin zymography performed on conditioned medium demonstrated that recombinant EDA (1 μM) strongly induced pro-MMP-9 expression (Fig. 1A). Recombinant EDB at similar concentrations caused only a modest increase in pro-MMP-9 release, whereas recombinant III11 domain, a 7-kDa recombinant protein representing the C terminus of repeat III1 (III1C), poly-l-histidine, and human...
Concentrations of recombinant EDA as low as 300 nM were detectable within 24 h (Fig. 2). A shorter time course revealed a 72 h incubation period with a marked increase in pro-MMP-9 concentration. Recombinant EDA, but not III11 or EDB, produced a response of the reporter construct in TLR4/MD-2 transfectants that was comparable to that of LPS (Fig. 6). The mechanism underlying the serum effect on EDA action is not known but could reflect the presence of an EDA-binding protein or antagonist. Importantly, the actions of LPS and recombinant EDA on IL-10 production were significantly blunted in C3H/HeN splenocytes, consistent with an action of LPS and EDA via Tlr4.

Using stable TLR4/MD-2 HEK 293 transfectants, we found that the LPS antagonist, E5531, inhibited LPS-stimulated MMP-9 production but did not block the action of EDA. THP-1 cells (5 × 10^5 cells/well) were cultured for 24 h in the presence of LPS (200 ng/ml), 256 nM E5531, EDA (300 nM), or a combination of LPS + E5531 or EDA + E5531 as indicated. Zymography was carried out on conditioned medium to demonstrate gelatinase activity.

NF-κB participates in the induction of MMP-9 gene transcription (24), and NF-κB is activated in response to LPS stimulation of TLR4. We found that components of NF-κB were increased in the nuclei of THP-1 cells as early as 15 min after addition of EDA, reaching peak levels at 1 h that were maintained up to 3 h (Fig. 4).

To determine whether recombinant EDA can activate TLR4, we transfected HEK 293 cells, which do not normally express TLR4, with expression plasmids for TLR4 and its accessory proteins and the reporter construct, pELAM-1-luc, which contains a fragment of the NF-κB-responsive E-selectin promoter coupled to luciferase (14) and then challenged the transfected cells with recombinant fibronectin domains or LPS. LPS in the presence of sCD14, which binds LPS and presumably presents it to TLR4, produced the expected strong reporter response in cells transfected with TLR4 and MD-2 (14, 15) but not in cells that did not receive the TLR4 expression plasmid (Fig. 5). Recombinant EDA, but not III11 or EDB, produced a response of the reporter construct in TLR4/MD-2 transfectants that was 40–45% of the LPS response. Like LPS, cotransfection with TLR4 and MD-2 was required for a vigorous response of the reporter construct to EDA (Fig. 6).

To further verify that recombinant EDA activates TLR4, we isolated splenocytes from C3H/HeJ mice, which have an activating mutation in the Tlr4 gene, and C3H/HeN control mice and challenged them with LPS, recombinant EDA, EDB, and III11 and measured IL-10 production. LPS (100 ng/ml) and EDA (1 μM) stimulated IL-10 production by C3H/HeN splenocytes, whereas recombinant EDB and III11 had minimal stimulatory effects (Table I). The relative activity of EDA (1 μM) compared with 100 ng/ml LPS in stimulating IL-10 release by C3H/HeN splenocytes was somewhat lower than the relative response that we observed in activation of TLR4 in the HEK 293 cells. This may be because of differences in ligand specificity between the human and murine TLR4s (e.g., taxol activates murine Tlr4 but not human TLR4) or to the presence of a higher concentration of serum (10% FBS) in the splenocyte assay compared with 0.5% FBS in the HEK 293 studies (and serum-free conditions with THP-1 cells). We have found that serum blunts the response to recombinant EDA but not to LPS. Therefore, the mechanism underlying the serum effect on EDA action is not known but could reflect the presence of an EDA-binding protein or antagonist. Importantly, the actions of LPS and recombinant EDA on IL-10 production were significantly blunted in C3H/HeN splenocytes, consistent with an action of LPS and EDA via Tlr4.

Using stable TLR4/MD-2 HEK 293 transfectants, we found that LPS in the absence of sCD14 had a minimal effect on TLR4-mediated reporter gene activation (Fig. 7), but there was a dramatic increase in the response to LPS when sCD14 was added. In the absence of sCD14, polymyxin B and the LPS antagonist, E5564, had no major impact on EDA activation of TLR4. However, the same concentrations of polymyxin B and E5564 completely blocked the stimulatory effects of LPS in the presence of sCD14. sCD14 (10 ng/ml) also increased the response to recombinant EDA (Fig. 7). This sCD14 augmentation of EDA activation of TLR4 was inhibited by polymyxin B and E5564, raising the possibility that the increased activity of EDA in the presence of sCD14 could be due, in part, to LPS contamination. Heat treatment ablated the
TLR4-stimulating activity of recombinant EDA but had no effect on LPS. Collectively, these experiments demonstrate that EDA activation of TLR4 cannot be accounted for by LPS contamination in the recombinant protein preparations.

Other investigators have examined the effects of fibronectin as well as fibronectin fragments not containing EDA on various cells and concluded that the responses are a consequence of fibronectin binding to integrin fibronectin receptors (25–27). Fibronectin fragment effects have also been linked to cellular differentiation, adhesion, and changes in the organization of actin filaments (25–27). Our observations suggest an alternative signaling pathway for fibronectin molecules containing the EDA domain.

The known TLR4 activators require accessory proteins to turn on TLR4 signaling. Like LPS, EDA activation of TLR4 requires MD-2, suggesting shared features of the mechanism of TLR4 activation. LPS requires CD14 for efficient activation of TLR4. Although we found that sCD14 enhanced the EDA activation of TLR4, we cannot rule out the possibility that sCD14 exposed the activity of minor LPS contaminants. It is of interest that Asea et al. (28) recently found that hsp 70 acts as a cytokine employing a CD14-dependent pathway, suggesting that CD14, a known lipid-binding protein, can also augment the action of proteins. We have been unable to demonstrate binding of EDA to sCD14. Therefore, if CD14 does increase the action of EDA, it must do so through a mechanism that does not involve high affinity interactions between these molecules. Importantly, polymyxin B and LPS antagonists did not have a major impact on EDA-stimulated THP-1 cell MMP-9 production and EDA activation of TLR4. The activity of EDA was destroyed by heat whereas LPS activity was unaffected. Thus, the effects of the EDA domain can be clearly distinguished from those of LPS.

Saito et al. (4) recently reported that recombinant EDA domains, but not intact cellular fibronectin, induce proinflammatory cytokines and MMP expression by rabbit synovial cells. Likewise, we found that intact cellular fibronectin did not have a major impact on MMP-9 expression in THP-1 cells. Saito et al. (4) also isolated a fibronectin fragment from human placenta containing the EDA domain in its C terminus. This

\[ ^2 \text{Y. Okamura and J. F. Strauss, unpublished data.} \]
fibronectin fragment displayed activities similar to that of the recombinant EDA domains. Their observations lend credence to the notion that endogenously generated EDA-containing fibronectin fragments serve as signaling molecules and indicate that domains in the fibronectin protein distal to the EDA domain may suppress the ability of fibronectin to activate signaling. Further investigation is needed to elucidate 1) the mechanism by which EDA stimulates TLR4 activity and 2) the structure of endogenous EDA-containing fibronectin fragments.

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FIG. 7. Effects of heat treatment, polymyxin B, and the LPS antagonist, E5564, on EDA activation of TLR4. HEK-TLR4/MD2/ELAM-luc cells were seeded in 96-well plates and maintained as described under “Experimental Procedures.” Cells were incubated with EDA (1 μM) or LPS (10 ng/ml) in the absence or presence of 10 nM sCD14 with phosphate-buffered saline (PBS), 10 μg/ml polymyxin B (Polymix), or 1 μM E5564 as indicated for 18 h. Heat indicates that EDA and LPS were heated (95 °C for 60 min) before addition to the cells. Following the incubation period, cell lysates were immediately analyzed for luciferase activity. Values are means ± S.D. from quadruplicate cultures from a representative experiment.
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J. Biol. Chem. 2001, 276:10229-10233.
doi: 10.1074/jbc.M100099200 originally published online January 9, 2001

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