Yersinia enterocolitica Promotes Deactivation of Macrophage Mitogen-activated Protein Kinases Extracellular Signal-regulated Kinase-1/2, p38, and c-Jun NH₂-terminal Kinase

CORRELATION WITH ITS INHIBITORY EFFECT ON TUMOR NECROSIS FACTOR-α PRODUCTION⁎

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The enteropathogenic bacterium Yersinia enterocolitica counteracts host defense mechanisms by interfering with eukaryotic signal transduction pathways. In this study, we investigated the mechanism by which Y. enterocolitica prevents macrophage tumor necrosis factor-α (TNFα) production. Murine J774A.1 macrophages responded to Y. enterocolitica infection by rapid activation of mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (ERK), p38, and c-Jun NH₂-terminal kinase (JNK). However, after initial activation, the virulent Y. enterocolitica strain gradually suppressed phosphorylation of the transcription factors Elk-1, activating transcription factor 2 (ATF2), and c-Jun, indicating time-dependent inhibition of ERK1/2, p38, and JNK kinase activities, respectively. Analysis of different Y. enterocolitica mutants revealed that (i) MAPK inactivation parallels the inhibition of TNFα release, (ii) the suppressor effect on TNFα production, which originates from the lack of TNFα mRNA, is distinct from the ability of Y. enterocolitica to resist phagocytosis and to prevent the oxidative burst, (iii) the tyrosine phosphatase YopH, encoded by the Y. enterocolitica virulence plasmid, is not involved in the decrease of ERK1/2 and p38 tyrosine phosphorylation or in the cytokine suppressive effect. Altogether, these results indicate that Y. enterocolitica possesses one or more virulence proteins that suppress TNFα production by inhibiting ERK1/2, p38, and JNK kinase activities.

The enteropathogenic Gram-negative bacterium Yersinia enterocolitica has developed strategies to resist the host immune defense. This enables extracellular survival and multiplication of the bacteria in host lymphoid tissue after infection and invasion of the intestinal mucosa. It is becoming increasingly evident that Yersinia sp. evade host defense mechanisms by disrupting key functions of the host cell. This ability is linked to the expression of a set of released plasmid-encoded proteins, termed Yersinia outer proteins (Yops)1 (1, 2). Export of Yops is triggered by attachment of Yersinia sp. to the host cell (3–5). Eleven Yops have been described so far (2). At least four of them, i.e. YopE, YopH, YopM, and YopO (the homolog of YpkA in Yersinia pseudotuberculosis), are translocated across the host cell membrane to their putative intracellular targets (3, 4, 6–10). YopE disrupts actin filaments (3, 4, 11) and acts synergistically with the protein-tyrosine phosphatase YopH (12) to inhibit phagocytosis and to suppress the oxidative burst of professional phagocytes (11, 13–16). YopH and also YopO, which displays serine/threonine kinase activity (17), share homologies with eukaryotic proteins, and both are supposed to interfere or block host cell signal transduction pathways (12, 17–20).

Y. enterocolitica, like other pathogens (Brucella sp. (21, 22), Bacillus anthracis (23), or Leishmania donovani (24)), also interferes with cytokine production. It suppresses chemokine interleukin-8 secretion of epithelial cells (25) and prevents production of the macrophage proinflammatory cytokine TNFα (26–29). Released TNFα enhances the activation of cells involved in the immune defense (i.e. macrophages, polymorphonuclear leukocytes, NK cells, and T lymphocytes) and thus contributes in overcoming bacterial infection. Previous studies already demonstrated that TNFα also plays an important role in limiting the severity of Y. enterocolitica infection (30). However, the impact of Y. enterocolitica on signaling pathways of mammalian cells, leading to suppression of cytokine release, is still completely unknown. Since LPS itself stimulates macrophage secretion of TNFα (30), it seems reasonable to assume that Y. enterocolitica interferes with LPS-stimulated pathways.

LPS from Gram-negative bacteria was reported to activate the three different MAPK families, i.e. ERK, JNK/SAPK, and p38, in macrophages (31–35). The mechanism of MAPK activation by LPS remains unclear (36). On the one hand, ceramide

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1 The abbreviations used are: Yop, Yersinia outer protein; TNFα, tumor necrosis factor; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase (MAPK is used here in a general sense and includes ERK, JNK, and p38 kinases); ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase (also termed SAPK); SAPK, stress-activated protein kinase; p38, murine homologue of the Saccharomyces cerevisiae high osmolarity glycerol protein kinase HOG1; LPS, lipopolysaccharide of Gram-negative bacteria; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; β₂m, β₂-microglobulin; PMSF, phenylmethylsulfonyl fluoride; MEF, MAPK/ERK kinase; ATP2, activating transcription factor 2.
seems to play an important role (37), since LPS was shown to activate a ceramide-dependent kinase (38) and ceramide itself stimulates the JNK/SAPK pathway (39). Moreover, c-Raf, the upstream kinase activator of MEK1/2 and ERK1/2, was reported to stimulate the JNK/SAPK pathway (39). Moreover, c-Raf, the upstream kinase activator of MEK1/2 and ERK1/2, was reported to stimulate the JNK/SAPK pathway (39).

In the present study, we analyzed possible alteration of MAPK activation during infection with Y. enterocolitica. We thus chose the macrophage-like J774A.1 cell line as a well established infection model to study Yersinia sp. macrophage interactions (13–14, 18–19, 43). Interestingly, there is a non-virulent Y. enterocolitica strain that is virulence plasmid-cured, thus providing an ideal control for comparison experiments with virulent type or mutated Y. enterocolitica strains (Table I). Here, we report that virulent Y. enterocolitica indeed strongly interferes with macrophage signal transduction, resulting in blockade of ERK, JNK, and p38 MAPK activities. This MAPK inhibition correlates with the suppression of TNFa production but is not required for the inhibition of macrophage phagocytosis and oxidative burst.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth, and Infection Conditions—The bacterial strains used in this study are listed in Table I. Overnight cultures grown at 26 °C were diluted 1:20 in fresh Luria-Bertani broth and grown for 2 h at 37 °C as described previously (16). Bacteria were then washed once and resuspended in phosphate-buffered saline. Cells were infected at a ratio of 50 bacteria/cell. The desired bacterial concentrations were adjusted by measuring the optical density at 600 nm and by adding 30 mM Na3VO4, 0.5 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 20 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 0.1 mM Na2VO4, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 0.5 mM PMSF. For immunoprecipitation, cell lysates were incubated with polyclonal anti-p38 antibody at 4 °C for 1 h. Immunocomplexes were then collected with protein A-Sepharose (Pharmacia Biotech, Up-sala, Sweden) and washed three times with radioimmune precipitation buffer. Both whole cell lysates and immunoprecipitates were mixed with 4 × or 2 × Laemmli buffer, respectively. Proteins were separated by 10% SDS-PAGE, electrotransferred to PVDF membrane (Polycreen, DuPont NEN), blocked with 3% bovine serum albumin, and probed with appropriate antibodies. Phosphotyrosine immunostaining was performed with the monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Immunoblotting for p38 and ERK1/2 was performed using rabbit polyclonal anti-p38 antibodies (1:3000 dilution) or goat polyclonal anti-ERK1/2 antibodies (1:30,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively. Immunoreactive bands were visualized by incubation (1 h) with rabbit anti-mouse (1: 10,000 dilution, Sigma), goat anti-rabbit (1:20,000 dilution, Sigma), or rabbit anti-goat (1:10,000, Santa Cruz Biotechnology) antibodies conjugated to horseradish peroxidase using enhanced chemiluminescence reagents (Renaissance; DuPont NEN). When required, membranes were stripped in 62.5 mM Tris, pH 6.7, 0.1 mM 2-mercaptoethanol, and 2% SDS for 30 min at 50 °C after film exposure. Thereafter, membranes were reprobed with appropriate primary and secondary antibodies and developed by chemiluminescence.

MAPK Assays—5 × 10⁶ cells treated with bacteria or LPS for different periods of time were lysed in 200 μl of cell extract buffer (25 mM Hepes, pH 7.7, 0.3 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na2VO4, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml benzamidin, 2 μg/ml pepstatin, 100 μg/ml PMSF). After centrifugation, the supernatant was diluted with 600 μl of cell extract buffer (20 mM Hepes, pH 7.7, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na2VO4, 2 μg/ml leupeptin, 10 μg/ml benzamidin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 100 μg/ml PMSF) and incubated on ice for 10 min, and centrifuged again. Lysates were then mixed with GST fusion protein kinase substrates (8 μg of each, as indicated) and glutathione-agarose (20 μl, Sigma) and incubated overnight at 4 °C. Experiments were performed as described (48) with four different GST fusion protein kinase substrates obtained from M. Karin (GST-c-Jun–1–222), B. Dérjard (GST-c-Jun–1–79) and GST-AFF2, and A. Nordheim (GST-Elk-1). The substrate-agarose complexes were washed four times with binding buffer (20 mM NaCl, pH 7.7, 50 mM NaCl, 25 μM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100), and in vitro phosphorylation was carried out for 20 min at 30 °C in the presence of 20 μl of Hepes, pH 7.6, 20 mM MgCl2, 2 mM dithiotreitol, 20 mM β-glycerophosphate, 0.1 mM Na2VO4, 20 mM p-nitrophenol, 20 μM ATP (4 μCi of [γ-32P]ATP) (total volume, 30 μl). The reaction was stopped by a single wash with binding buffer and by adding 30 μl of 2 × Laemmli buffer. Proteins were fractionated by 10% SDS-PAGE, electrotransferred to PVDF membrane, and sub-

### Table I

| Strain                              | Relevant characteristics | Former designation | Reference |
|-------------------------------------|--------------------------|--------------------|-----------|
| **Virulent**                        |                          |                    |           |
| Serogroup O8; clinical isolate harboring virulence plasmid pYVO8 | WA-314                  | 44                  |
| **Nonvirulent**                     |                          |                    |           |
| Plasmidless derivative of the virulent strain | WA-C                    | 44                  |
| **YopH(1)**                         |                          |                    |           |
| Mutant strain, deficient in YopH secretion; insertional inactivation of sycH, the gene for the YopH-specific chaperone SycH | WA-(pYVO 08:Tn7)        | 45                  |
| **YopH(1)/H**                       |                          |                    |           |
| YopH(1)– strain, complemented with sycH and yopH; YopH secretion-positive | WA-(pYVO 7146)          | 16                  |
| **YopH(2)−**                       |                          |                    |           |
| YopH mutant; insertional inactivation of the yopH gene | WA-(pYV 08A)H          | 46                  |
| **Yop sec.−**                       |                          |                    |           |
| Mutant strain, deficient in secretion of Yops; insertional inactivation of lcrD, the gene encoding LcrD, which is essential for Yop secretion | WA-(pYV 515)            | 16                  |
| **YopD,B,N,V+**                     |                          |                    |           |
| Strain harboring plasmid pLCR encoding the secretion apparatus of Y. enterocolitica including the genes for YopD, YopB, YopN, and the V antigen | WA-(pLCR)               | 46                  |
| **YopD,B,N,V, YadA−**               |                          |                    |           |
| Strain YopD,B,N,V harboring an additional plasmid encoding the genes for YopH, YopE, and YadA | WA-(pLCR,pB−23)         | 46                  |

Y. enterocolitica Inhibits TNFa Release by MAPK Deactivation

Yersinia enterocolitica virulent wild-type or mutated Y. enterocolitica strains used in this study are listed in Table I. Antibodies directed against the C-terminal end (peptide KPLDQEEMES) of p38 kinase were raised in New Zealand rabbits as described previously (47). Antibodies were purified from immune sera by ammonium sulfate precipitation followed by overnight recycling through Affi-Gel 10 (Bio-Rad) to which the antigen peptide had been linked. After acidic elution, neutralized affinity-purified antibodies were diazylated against phosphate-buffered saline and thereafter against a glycerol/phosphate-buffered saline solution (1:1) before storage at −20 °C.
Y. enterocolitica Inhibits TNFα Release by MAPK Deactivation

**RESULTS**

**Y. enterocolitica Reduces p38 and ERK1/2 Tyrosine Phosphorylation and MAPK Activities**—To determine possible differences between the virulent and nonvirulent Y. enterocolitica strain during infection, we compared the patterns of tyrosine-phosphorylated proteins in cells stimulated with LPS from E. coli or with the two Y. enterocolitica strains. Cell lysates prepared after different stimulation times were immunostained with the monoclonal anti-phosphotyrosine antibody 4G10. After 15 min of stimulation with LPS, nonvirulent and virulent Yersinia, there was a dramatic increase in tyrosine phosphorylation of two proteins at the 38- and 42-kDa level (Fig. 1A). The phosphorylated 38-kDa protein was shown, by stripping and reprobing the membrane with the anti-p38 peptide antibody, to have the same electrophoretic mobility as p38, also known as RK, reactivating kinase (52, 53), or for cytokine-suppressive anti-inflammatory drug binding proteins (41) (data not shown). Repробing the membrane in the same manner with a pan-anti-ERK antibody demonstrated that the tyrosine-phosphorylated protein at 42 kDa was ERK2 (Fig. 1B). This immunoblot also allowed us to distinguish between the unphosphorylated and phosphorylated forms of ERK2 and ERK1, since the phosphorylated forms exhibited slower electrophoretic mobilities. In unstimulated cells (Fig. 1B, lane 4), ERK labeling corresponded to unphosphorylated forms of ERK2 (lower band) and ERK1 (upper band). Cell treatment with LPS and Y. enterocolitica strains induced a total upward shift in the ERK proteins, in accordance with the strong tyrosine-phosphorylation of ERK2 at about 42 kDa in panel A. It was not clear from panel A whether or not ERK1 (44 kDa) was also phosphorylated, because of intense phosphotyrosine labeling at about 44 kDa that was not regulated by LPS or bacterial stimulation. Nevertheless, Fig. 1B revealed a electrophoretic shift that also occurred at the ERK1 level, demonstrating that ERK1/p44MAPK was phosphorylated over the same time course as ERK2. We thus referred to these proteins as ERK1/2, since both ERK proteins behaved similarly. Interestingly, in the

![FIG. 1. Time course of tyrosine phosphorylation of J774A.1 cells treated with Y. enterocolitica or LPS.](image-url)
strain (Fig. 2, lanes 3 with LPS (Fig. 2, ERK and p38, respectively (48), c-Jun appears to be specifically cannot be considered as selective substrates for the kinases Elk-1, ATF2 and c-Jun (Fig. 2). Although Elk-1 and ATF2 cytosolic extracts to phosphorylate the transcription factors. This phenomenon, i.e. interference of Yersinia sp. with macrophage tyrosine phosphorylation, has already been described (18, 19, 54, 55) and can at least partially be attributed to the tyrosine phosphatase of Yersinia sp., named YopH. In any case, the virulent Y. enterocolitica strain selectively decreased p38 and ERK1/2 tyrosine phosphorylation levels, indicating that their kinase activities, conferred by dual phosphorylation of the Thr-X-Tyr motif, should be concomitantly reduced.

To directly measure the activities of ERK and p38 and to determine whether JNK activity was also affected by the inhibitory effect of Y. enterocolitica, we analyzed the ability of cytosolic extracts to phosphorylate the transcription factors Elk-1, ATF2 and c-Jun (Fig. 2). Although Elk-1 and ATF2 cannot be considered as selective substrates for the kinases ERK and p38, respectively (48), c-Jun appears to be specifically phosphorylated by JNK. A 60-min incubation of J774A.1 cells with LPS (Fig. 2, lanes 2) or the nonvirulent Y. enterocolitica strain (Fig. 2, lanes 3) induced a substantial (3–10-fold) increase in phosphotransferase activities toward the different GST fusion proteins, including the two GST-c-Jun substrates. This clearly indicated that Y. enterocolitica also stimulated kinase activity of the JNK protein. However, after infection with the virulent Y. enterocolitica strain (Fig. 2, lanes 4), phosphorylation of all substrates was markedly reduced as compared with the non-virulent strain, since the substrate phosphorylation was only 1.5–3-fold that of the control level. The parallel alteration of the different GST fusion proteins, including the two GST-c-Jun substrates, indicated that in addition to the reduction in ERK and p38 kinase activities, the virulent Y. enterocolitica strain also inhibited JNK activity, as revealed by the weaker phosphorylation of both GST-c-Jun-(1–79) and GST-c-Jun-(1–222) substrates.

Inhibition of TNFα Production Is Associated with a Reduction in MAPK Activities—Since the virulent Y. enterocolitica strain abolished MAPK activation, we wondered whether MAPK deactivation was related to the inhibition of macrophage TNFα secretion. Figure 3 (lanes 2–4) confirms that LPS and nonvirulent yersiniae induced a strong TNFα-response in J774A.1 cells, while the virulent Y. enterocolitica strain, which prevented p38 and ERK1/2 tyrosine phosphorylation, completely blocked TNFα secretion (Fig. 3A) as well as TNFα mRNA expression (Fig. 3B). Furthermore, when cells were first infected with the virulent Y. enterocolitica strain, further stimulation with LPS from E. coli could trigger neither TNFα production nor reduction of p38/ERK1/2 tyrosine phosphorylation. These two properties were compared with untreated cells (lane 1) or cells treated with LPS (lane 2), the nonvirulent Y. enterocolitica strain (lane 3), the virulent Y. enterocolitica strain (lane 4), the YopH(+) strain (lane 5), the Yop secre. strain (lane 6), the YopD,B,N,V,H,YadA strain (lane 7), or the YopD,B,N,V,H,E,YadA strain (lane 8) (for description of the strains, see Table 1). A, TNFα-production; cells were untreated or treated with bacteria or LPS. After 60 min of infection, extracellular bacteria were killed with gentamicin, and the TNFα activity of the cell culture supernatant was measured after a final incubation time of 120 min, using a cytotoxic assay performed with the TNFα-sensitive fibroblast cell line L929. B, reverse transcriptase-PCR detection of TNFα mRNA. Total RNA was isolated from cells treated as described above. RNA was reverse transcribed. The PCR products for TNFα and βm obtained after 20 cycles were analyzed on agarose gel. Results shown are representative of two independent experiments. C, p38/ERK2 tyrosine phosphorylation; cells were treated with bacteria and/or LPS and lysed at the times indicated. In the bottom panel, cells treated for 90 min, as indicated, were challenged with LPS for another 30 min. Lysates were subjected to SDS-PAGE and immunoblotted with the anti-phosphorysine antibody 4G10. Only the relevant part of each immunoblot displaying the levels of tyrosine phosphorylation of ERK2 and p38 is shown.
production (data not shown) nor tyrosine rephosphorylation of p38 and ERK2 (Fig. 3C, bottom panel). To gain further insight into a possible relation between the lack of TNFα production and the decreased p38 and ERK1/2 tyrosine phosphorylation, we analyzed defined Y. enterocolitica mutants. The YopH(1) strain, a mutant with selectively impaired secretion of the protein-tyrosine phosphatase YopH, prevented tyrosine phosphorylation and TNF production to a similar extent as the virulent wild-type strain (Fig. 3, lanes 4 and 5). On the contrary, the Yop secretion-negative LcrD−/− mutant with defective secretion of all Yops (Yop secre., Fig. 3, lane 6), did not decrease p38 and ERK2 tyrosine phosphorylation and induced strong TNFα release, similar to the nonvirulent strain. Fig. 3 also shows the results obtained with two other mutants expressing a restricted repertoire of yop genes. The YopD,B,N,V−/− strain harbors the fragment of the Y. enterocolitica virulence plasmid encoding the Yop secretion machinery, including the genes coding for YopD, YopB, YopN, and the V antigen, which are necessary for Yop expression, secretion, and translocation. The second strain, referred to as YopD,B,N,V,H,E,YadA−/−, expressed, in addition to yopD, yopB, yopN, and lcrV (encoding the V antigen), yopH and yopE, which encode the translocated proteins YopH and YopE, and yadA, encoding the cell adhesin YadA. Analysis of these two mutants indicated that they were able neither to reduce p38 and ERK1/2 phosphorylation nor to block TNFα-production of J774A.1 cells (Fig. 3, lanes 7 and 8).

To compare the action of various mutants on MAPK activities, a time course study was performed with GST-Elk-1, GST-ATF2, and GST-c-Jun (Fig. 4) as in Fig. 2, except that the three substrates were added together in the kinase assay. In agreement with the phosphorylation of p38 and ERK1/2 seen in Fig. 3B, LPS of E. coli and all Y. enterocolitica strains induced strong phosphorylation of the three substrates within 30 min of stimulation (Fig. 4). Thereafter, only cells infected with the virulent Y. enterocolitica strain and with the YopH(1)−/− mutant exhibited almost complete disappearance of kinase activities within 60–90 min. The reduction in phosphorylation occurred over a similar time course for the three substrates, suggesting that the virulent and the YopH(1)−/− strain decreased the activities of MAPK cascades simultaneously. MAPK activities were also inhibited within 90 min, when virulent yersiniae were killed after 30 min of infection by the addition of 100 μg/ml gentamicin (data not shown). Taken together, these results indicate the existence of a relation among blockade of p38/ERK1/2 tyrosine phosphorylation, inhibition of p38/ERK1/2/JNK kinase activities, and suppression of TNFα-production.

**Table II**

| Y. enterocolitica strain | TNFα releasea | Phagocytosisb | Oxidative burstc |
|-------------------------|---------------|---------------|-----------------|
| Nonvirulent             | 87 ± 2        | 91 ± 1        | 65 ± 2          |
| Virulent                | 5 ± 1         | 5 ± 1         | 5 ± 4           |
| YopD,N,V−/−             | 64 ± 6        | 87 ± 3        | 58 ± 16         |
| YopD,B,N,V,H,E,YadA−/−  | 97 ± 10       | 5 ± 2         | 5 ± 5           |

aResults for TNFα release are the values from the experiment depicted in Fig. 3, expressed as percentages of released TNFα inducing 10 μg/ml LPS (551 ± 20 pg/ml = 100%).

bCells were incubated with bacteria for 1 h and then stained by a double-immunofluorescence technique to discriminate between intracellularly located bacteria. Mean percentages of ingested bacteria with respect to the total number of bacteria per cell were determined by counting 100 cells from each experiment.

cCells were preexposed to Y. enterocolitica for 90 min and then treated with opsonized zymosan. Chemiluminescence responses were recorded for a total of 30 min. Mean percentages of the zymosan-induced chemiluminescence response of cells not preexposed to bacteria (100%) are shown. Values ± S.E. shown in columns three and four are from three independent experiments.

The Virulent Y. enterocolitica Strain Reduces Raf-1 Kinase Activity—Since the reduction in MAPK tyrosine phosphorylation and activation cannot be attributed to the phosphatase
phages were infected with the virulent Y. enterocolitica strain (lane 4), the YopH(1)− strain with defective secretion of YopH (lane 5), the YopH(1)−/H+ strain complemented and secreting YopH (lane 6), the YopH(2)− strain with defective expression of YopH (lane 7), or the Yop sec− strain with defective secretion of all Yops (lane 8). Thereafter, cells were restimulated with LPS for 30 min (lanes 2–8). After cell lysis, p38 was immunoprecipitated with polyclonal anti-p38 antibodies, subjected to SDS-PAGE, and immunoblotted with the anti-phosphotyrosine antibody 4G10, as described under “Experimental Procedures.” The bands stained above the p38 protein correspond to nonspecific labeling of chains of rabbit anti-p38 antibodies used in the immunoprecipitation procedure.

YopH, we analyzed Raf-1 kinase activities to determine whether the virulent Y. enterocolitica strain modulates signaling pathways upstream of MAPKs. Fig. 6 demonstrates that the ability of Raf-1 to activate MEK1, which then in turn activates MEK upstream of MAPKs. Fig. 6 demonstrates that the ability of Raf-1 to activate MEK1, which then in turn activates MEK upstream of MAPKs.

DISCUSSION

Yersinia sp., like a number of other microbial pathogens, are supposed to modulate eukaryotic signaling pathways for their own benefit (20). In this study, we analyzed the impact of Y. enterocolitica on macrophage MAPK signaling pathways using J774A.1 cells as an infection model. Infection with Y. enterocolitica was found to stimulate p38 and ERK1/2 MAPK pathways, as detected by tyrosine phosphorylation. By contrast, direct tyrosine phosphorylation of JNK was not obvious, but this has been attributed to the fact that JNK phosphorytrosine cannot be easily detected by immunoblotting using an antiphosphotyrosine antibody (56, 57). The patterns of tyrosine-phosphorylated proteins (Figs. 1 and 3) and kinase activities (Figs. 2 and 4) obtained with LPS and virulent and nonvirulent Y. enterocolitica strains were very similar over the first 30 min. Thereafter, the virulent strain harboring the Y. enterocolitica virulence plasmid induced a substantial reduction in kinase activities, as revealed by a decrease in the phosphorylation of ERK1/2 and p38 kinases along with their substrates, the transcription factors Elk-1 and ATF2, as well as that of the JNK-specific substrate c-Jun. The fact that the reduction in kinase activities occurred after only 1 h of cell infection can be explained by the delay necessary for the Yops to reach their targets and to exert their effects on the host cell (5, 16, 19). The initial stimulation of the three types of MAPKs followed by selective inhibition with the virulent Y. enterocolitica strain was also observed with macrophages derived from human monocytes (data not shown).

A link between MAPK activation and TNFα production induced by LPS has been widely documented (41–42, 58–60). Deactivation of the MAPKs p38, JNK, and ERK1/2 induced by virulent Y. enterocolitica, therefore, might be related to its inhibitory effect on macropage TNFα secretion. Indeed, we found that all investigated Y. enterocolitica strains capable of inhibiting MAPK activities also prevented TNFα production, and reciprocally, all strains inhibiting TNFα release also deactivated MAPKs. This finding strongly supports the hypothesis that inhibition of TNFα release by Y. enterocolitica originates from shortening p38, ERK1/2, and JNK activation by reducing their levels of tyrosine phosphorylation. Furthermore, our evaluation of TNFα mRNA levels by reverse transcriptase-PCR indicated that LPS stimulation and infection with the nonvirulent Y. enterocolitica strain dramatically enhanced the amount of TNFα mRNA, while no accumulation of this messenger occurred after infection with the virulent strain. This indicates that the inhibitory effect of the virulent Y. enterocolitica strain on TNFα release is probably not due to alteration of TNFα maturation or secretion but rather to a lack of TNFα mRNA accumulation. The absence of mRNA may be due to inhibition of TNFα gene transcription or due to mRNA instability. A role of p38 in post-transcriptional control of TNFα gene expression has been clearly shown by the group of Lee (41, 42) using the anti-inflammatory drug SB203580. It is thus possible that the accelerated dephosphorylation of p38 is partially responsible for the inhibition of TNFα synthesis.

The fact that not only p38, but also ERK1/2 and JNK, are deactivated by the virulent Y. enterocolitica strain raises the question of their potential role in TNFα suppression. Indeed, it was recently shown that blockage of the ERK pathway by the MEK inhibitor PD98059 prevents TNFα mRNA synthesis induced by FcγR stimulation (58). No specific drugs for the JNK pathway are available yet. However, TNFα gene expression is stimulated by AP-1 (61, 62), a transcription factor composed of c-Jun and c-Fos, which are activated through phosphorylation by ERK and JNK (63). Deactivation of p38, ERK, and JNK induced by the virulent Y. enterocolitica strain may thus to-
gether contribute to inhibition of TNFα synthesis. The similar time courses of deactivation of all the MAPK, ERK1/2, JNK, and p38 suggests that yet unidentified bacterial virulence factors might act at a step that is common to the three pathways, i.e. upstream of MAPKs. When investigating this possibility, we found that Raf-1 activity was lowered after infection with the virulent Y. enterocolitica strain compared with the nonviral ser strain. This finding indicates that at least part of the TNFα-inhibitory action takes place upstream of the MAPKs. However, it remains to be determined whether Y. enterocolitica inhibits MAPK signaling cascades via reduction of upstream kinase activities only or whether it also causes dephosphorylation of MAP kinases themselves. It cannot be ruled out that bacterial factors trigger or accelerate the expression of an endogenous macrophage phosphatase, such as the specific MAPK phosphatase-1 (64, 65) or HVH1 or HVH2, two human homologs of the vaccinia virus dual specific phosphatase VH1 (66, 67).

In an attempt to identify the potential virulence factors involved in MAPK deactivation and TNFα inhibition, we compared the characteristics of several Y. enterocolitica mutants. Analysis of mutants with impaired tyrosine phosphatase YopH expression or secretion clearly excluded participation of YopH in the inhibitory effect on MAPK tyrosine phosphorylation and TNFα production. Furthermore, experiments on a mutant with defective secretion of all Yops (LcrD mutant) demonstrated that indeed one or several released Y. enterocolitica proteins other than YopH mediate inhibition of MAPK activities and TNFα production. Analysis of Y. enterocolitica strains capable of producing individual virulence factors revealed, in agreement with our previous data on granulocytes (16), that YopH and YopE confer resistance to phagocytosis and suppression of the J774A.1 cell oxidative burst by Y. enterocolitica; a strain, capable of producing YopH, YopE, and the adhesin YadA, as well as YopD, YopB, YopN, and the V antigen, suppressed macrophage phagocytosis and oxidative burst, in contrast to a strain secreting only the latter proteins, which are necessary for expression, secretion, and translocation of active Yops (2, 4, 7–8, 10). The fact that both strains were unable to inhibit MAPK activities and TNFα production clearly demonstrates that the anti-TNFα effect is not a consequence of the ability of Y. enterocolitica to inhibit phagocytosis and to suppress the oxidative burst. Furthermore, this finding implies that the Yops released by this strain (YopD, YopB, YopN, YopH, YopE, V antigen) are not, or at least not solely, responsible for the inhibition of TNFα production, although such an effect was previously attributed to YopB (28) and the V antigen (26, 27).

In summary, we demonstrated for the first time that virulent Y. enterocolitica mediates disruption of eukaryotic signal transduction. Moreover, our study highlights a correlation between the inhibition of macrophage TNFα production by Y. enterocolitica and deactivation of MAPK pathways. The virulence factors responsible for these inhibitory effects are released Y. enterocolitica proteins other than YopH or YopE. The cellular target from which the different MAPK pathways are affected seems to be located at the MAPK kinase kinase level, i.e. Raf-1, or upstream. These characteristics point to the Ras superfamily of small G proteins, among which Cdc42, Rac, and Rho appear to be activated in cascade, with subsequent activation of multiple pathways including MAPK modules (68). Studies presently under way address this question and should provide new insight into the pathogenesis of yersiniosis.

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