Identification of a Bacterial Inhibitor of Protein Kinases

MECHANISM AND ROLE IN HOST CELL INVASION*

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We show that Escherichia coli produce a factor that inhibits the activity of tyrosine and serine/threonine protein kinases. The factor is a protein found in the periplasmic compartment and is also secreted into the culture medium. Using a particle concentration fluorescence immunoassay specific for tyrosine kinase activity and inhibition of the tyrosine kinase p56Lck, we purified this factor to apparent homogeneity. Analysis of trypsin-digested fragments by mass spectrometry identified the inhibitor as the bacterial periplasmic protein UDP-sugar hydrolase, an enzyme with potent and nonspecific 5'-nucleotidase activity. Overexpression of the enzyme in bacteria leads to coordinate increases in both 5'-nucleotidase and p56Lck inhibitory activity, confirming the identity of the inhibitor. The kinase inhibitory activity appears to be due to the formation of adenosine, which we show is inhibitory for p56Lck, cAMP-dependent protein kinase, and casein kinase. Overexpression of UDP-sugar hydrolase leads to an increase in the recovery of enteropathogenic E. coli following infection of HeLa cell monolayers and corresponding alterations in tyrosine-phosphorylated host proteins. These results suggest that UDP-sugar hydrolase may be an important factor affecting host cell function following intracellular bacterial infection.

A common element in the life cycle of many bacterial pathogens is the establishment of residence inside host cells (1, 2). Ligands and receptors mediating bacterial invasion have been identified, and, in some cases, aspects of host signal transduction that are required for the invasion process have been characterized (3–12). Less well known, however, are the factors influencing bacterial survival once inside the host cell. Some studies have documented gene expression differences for bacteria that reside inside host cells (13), and auxotrophic mutants of Salmonella have been found to be attenuated for pathogenicity (14–16). Using insertional mutagenesis in conjunction with large scale screening, several groups have identified mutants defective for invasion or intracellular replication (14, 17, 19). It is not clear, however, if any of the mutants act by producing a factor that inhibit the activity of bacterial invasion related protein kinase p56Lck (18). The key role that tyrosine kinases play in host cell signaling, coupled with the possibility that this unexpected bacterial activity might be used to interfere with these functions, prompted us to further investigate this factor. Here we describe the initial characterization, purification, and identification of this factor and show that Ser/Thr kinases are inhibited as well. We also demonstrate the involvement of this inhibitory activity in bacterial invasion of human cells.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains used in this study were DH5α (BRL), BL21 (Pharmacia, N4830-1 (Pharmacia Biotech Inc.) and EPECE2348/69 (20) (kindly provided by Dr. Brett Finlay, UBC, Vancouver). Bacterial cells were routinely grown in LB medium (Life Technologies, Inc.) at 28 °C or 37 °C with shaking. Ampicillin (Sigma), when added, was at 100 μg/ml. Plasmid pLA7 contains the UshA gene which encodes the UDP-sugar hydrolase protein in pBR322 (21). This plasmid was kindly provided by Dr. I Beacham (Griffith University, Brisbane, Australia). Plasmid pBR322 is from ATCC (37017).

Mammalian Cells—LSTRA cells are transformed murine T cells that overexpress p56Lck (22). They were obtained from Dr. Jamey Marth (San Diego). HeLa cells were from ATCC (CCL-2). Both cell lines were grown in RPMI (Life Technologies, Inc.) + 10% fetal bovine serum + penicillin and streptomycin. LSTRA cell cultures were also supplemented with 10–5 μM 2-mercaptoethanol.

Kinases—Two sources of p56Lck were used in this study. Baculovirus-expressed p56Lck was partially purified on a DEAE-column as described (23). Partially purified p56Lck from LSTRA cells was prepared as follows. 1 liter of cells was collected by centrifugation, washed with PBS, and resuspended in 15 ml of sonication buffer (20 mM MOPS, pH 7.2, 75 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM FMSF, 1 mM pepstatin). The cells were sonicated 4 times with 5-s pulses on ice, and nuclei and cell membranes were pelleted at 50,000 rpm for 20 min at 4 °C in a Beckman 70.1 Ti ultracentrifuge rotor. The pellet was resuspended in sonication buffer containing 1% Triton X-100 and recentrifuged at 70,000 rpm for 15 min at 4 °C in the same rotor. The supernatant containing partially purified p56Lck was aliquoted and stored at −80 °C until use.

cAMP-dependent protein kinase was purchased from Sigma. Casein kinase was purchased from UBI.

Kinase Assays—Tyrosine kinase activity and its inhibition was routinely assayed using a particle concentration fluorescence immunoassay (PCFIA) as described by Babcook et al. (24). Bovine brain myelin basic protein (Sigma) was covalently coupled to Fluoricon carboxyl-arylpolystyrene beads with EDC and used as a substrate. The beads were suspended in 20 mM Tris (pH 7.7), 0.5% BSA, 0.01% EvG-55 to a final concentration of 0.125% with an estimated MBP concentration of 25 μg/ml. Assay reactions were in 96-well Pandex assay plates in a total

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‡ The abbreviations used are: PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; FMSF, phenylmethylsulfonyl fluoride; PCFIA, particle concentration fluorescence immunoassay; MBP, myelin basic protein; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.
Bacterial Inhibitor of Protein Kinases

In vitro Kinase Assay—The ability of periplasmic fractions to inhibit p56\textsuperscript{ck} was also tested using an in vitro kinase assay. Baculovirus-expressed p56\textsuperscript{ck} was incubated in 20 mM Tris (pH 7.4) buffer with 0.5% BSA, 10 mM MnCl\textsubscript{2}, 10 mM DT, 0.5 mM unlabelled ATP and 100 μCi of γ\textsuperscript{32}P\textsuperscript{-}[\textsuperscript{33}P\textsuperscript{ATP} (50 μM ATP final concentration)] with the appropriate substrate (25 μg/ml MBP or 250 μg/ml partially dephosphorylated bovine casein (Sigma)). Reactions were initiated with the addition of ATP, incubated for 15 min at 30 °C, and then terminated by the addition of 5 μl EDTA. The reaction mixture was then bound to p81 phosphocellulose paper immobilized in a 96-well manifold. The filter paper was washed several times with 75 mM phosphoric acid, dried, and placed in a Packard "flexifilter" plate. Scintillation fluid was added to the wells and the plate was counted in a TopCount scintillation counter (Packard).

Inhibition of Tyrosine and Ser/Thr kinase activity was also assayed using incorporation of γ\textsuperscript{32}P\textsuperscript{-}[\textsuperscript{33}P\textsuperscript{ATP} into MBP containing 1% phosphotyrosine and immobilized on p81-phosphocellulose paper. Briefly, assays were performed in 50-μl volumes in assay buffer (20 mM Tris-Cl, pH 7.5, 10 mM MnCl\textsubscript{2}, or MgCl\textsubscript{2}, 1 mM dithiothreitol, 0.5% BSA, plus 10 μCi of γ\textsuperscript{32}P\textsuperscript{-}[\textsuperscript{33}P\textsuperscript{ATP} (50 μM ATP final concentration)] with the appropriate substrate (25 μg/ml MBP or 250 μg/ml partially dephosphorylated bovine casein (Sigma)). Reactions were initiated with the addition of ATP, incubated for 15 min at 30 °C, and then terminated by the addition of 5 μl EDTA. The reaction mixture was then passed through a Sephadex G-25 column (Pharmacia). Each sample buffer (10 mM Tris, pH 8, 1 mM EDTA, 0.01% Brij 35) for 10 column volumes, and run at 0.2 ml/min. 0.2-ml fractions were collected and analyzed for p56\textsuperscript{ck} inhibitory activity.

In order to further define this factor, the inhibitory activity of the monomer was analyzed by mass spectrometry as described by Hess et al. (25).

Assay for UDP-sugar Hydrolase—The enzymatic activity of UDP-sugar hydrolase was determined by measuring the hydrolysis of bis(p-nitrophenoxy) phosphate. Fractions containing UDP-sugar hydrolase were incubated in 0.1 mM Tris-Cl, pH 6.7, 5 mM MnCl\textsubscript{2}, 1 mg/ml bis(p-nitrophenoxy) phosphate (Sigma), 1 mg/ml BSA in a final volume of 100 μl in 96-well plates. After 20 min at 37 °C, the reaction was terminated with the addition of NaOH to a final concentration of 0.05 M and the release of p-nitrophenoxy was measured by absorbance at 405 nm.

TLC Analysis of UDP-sugar Hydrolase Products—Partially purified inhibitor fractions were incubated under standard kinase assay conditions with [\textsuperscript{33}P\textsuperscript{ATP} for 15 min at 37 °C. The reaction products were separated by thin layer chromatography on DEAE-cellulose plates with water/isobutyl alcohol/methanol/ammonium hydroxide in a ratio of 30: 10: 1: 0.1 (v/v) as the solvent (26). Location of the standards was determined by uv, and reaction products were visualized by autoradiography.

Invasion Assay—EPEC cells were grown overnight at 37 °C with shaking in LB with 100 μg/ml ampicillin. The overnight culture was diluted 1:20 and growth was continued for 2 h in LB + 1 mM MnCl\textsubscript{2}. At this point, typical A\textsubscript{600} were 0.2 to 0.3. The bacteria were collected, washed twice in PBS, and their concentration was adjusted to provide the appropriate multiplicity of infection. Aliquots of these cultures were plated on LB to estimate starting bacterial density. HeLa cells monolayers in 6-well plates were washed 3 times with RPMI + 10% fetal bovine serum containing 1% mannose, 1 mM MnCl\textsubscript{2} without antibiotic. The mannose was included to prevent nonspecific bacterial adherence to the monolayer. 0.2 μl of bacteria in 2 ml of medium was then added to the wells, and the plates were incubated at 37 °C for 1.5 h. The monolayers were washed 3 times with PBS and then 2 ml of medium containing 100 μg/ml gentamycin was added to kill all extracellular bacteria. Following a further 3-h incubation, the monolayers were again washed 3 times with PBS and lysed with 0.4 μl 1% Triton X-100 in PBS for 5 min. 1.6 ml of LB was added and aliquots were plated to measure recovered bacteria. Control experiments (not shown) demonstrated that the concentration of gentamycin used was sufficient to kill extracellular bacteria and that the Triton X-100 treatment did not affect bacterial plating efficiency. Control DH5α bacteria were found to be noninvasive in this assay.

Phosphotyrosine Content following EPEC Invasion of HeLa Cells—HeLa cell monolayers were lysed in 30 mM Tris (pH 6.8), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.3 mg/ml PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na\textsubscript{3}VO\textsubscript{4}. Lysates were analyzed for phosphotyrosine content by Western blotting with 4G10 anti-phosphotyrosine antibody as recommended by the supplier (UBI). The blot was developed and visualized with chemiluminescence reagents (Amersham).

RESULTS

Bacterial Lysates Contain an Activity That Inhibits p56\textsuperscript{ck}—In the course of using bacteria as hosts for recombinant protein expression, we found that bacterial lysates contain a substance capable of inhibiting the protein-tyrosine kinase p56\textsuperscript{ck}. Bacterial lysates were prepared from standard overnight cultures of N4830-1, DH5α, or BL21 cells grown in LB medium at 28 °C and lysed with Nonidet P-40. These lysates were then added to p56\textsuperscript{ck}-expressing E. coli. As shown in Fig. 1, untreated bacterial lysates from N4830-1 cells completely inhibited the ability of p56\textsuperscript{ck} to phosphorylate immobilized MBP. In order to further define this factor, the inhibitory activity of the monomer was analyzed by mass spectrometry as described by Hess et al. (25).
activity from periplasmic and cytoplasmic fractions were compared. Although both fractions contained inhibitory activity, most of the cytoplasmic inhibitory activity could be neutralized by adding protease or phosphatase inhibitors. In contrast, the inhibitory activity present in the periplasmic fraction could not be neutralized with these inhibitors.

We also found that the periplasmic inhibitory activity was moderately resistant to heat treatment but could be inactivated by incubating extracts for 1 h at 70 °C.

Phosphatases are present in the periplasm of bacteria grown under certain conditions such as low phosphate. As well, a *Yersinia* virulence factor has been shown to be a tyrosine-specific phosphatase (27, 28). We therefore tested to see if the inhibitor had tyrosine phosphatase activity using two methods. In the first method, MBP immobilized on beads was phosphorylated by p56<sup>Lck</sup>, the beads were washed and then incubated with periplasmic lysates. The phosphotyrosine remaining on the beads was then measured by PCFIA (24). No change in phosphotyrosine content was seen (not shown). In the second method, purified p56<sup>Lck</sup> was incubated with γ-labeled [32P]ATP and MBP with or without periplasmic extracts. The reaction products were then separated by SDS-PAGE, dried, and visualized by autoradiography. As shown in Fig. 2, when the periplasmic extract is included throughout the course of the reaction, no phosphate is incorporated into either p56<sup>Lck</sup> or MBP (lanes 6–8). When the periplasmic extract is added after the reaction has proceeded and phosphorylation has already occurred, incorporation ceases but does not decrease (lanes 2 and 4). These results suggest that the inhibitor is not a phosphatase, but rather interferes in some manner with the kinase activity of p56<sup>Lck</sup>.

Size exclusion chromatography was performed on periplasmic extracts, and fractions were assayed for inhibitory activity. As shown in Fig. 3, the inhibitory activity elutes in a single peak with a molecular weight of approximately 60,000.

**Purification and Properties**—The inhibitory activity proved to be stable for at least 1 week at 4 °C and for months at −80 °C; however, only limited freezing and thawing could be tolerated. Therefore, throughout the purification process, freeze-thaw cycles were limited to one or at most two.

Although the purification of the inhibitor was done with periplasmic extracts from N4830-1 cells, we subsequently found that the inhibitory activity was secreted as well and could be purified from culture supernatants.

A number of biochemical separations were used to purify the periplasmic inhibitor. A summary of the separations is shown in Fig. 4 and Table I. The effective steps were found to be ammonium sulfate precipitation, ion exchange chromatography on Q-Sepharose, elution from hydroxyapatite with NaF, isoelectric focusing, and further ion exchange separation on Mono Q. The combination of these steps results in an approximate 21,600-fold purification. Note that total recoverable activity increases following the first ion exchange step.

**Identification of the Inhibitor**—These results strongly suggested that the $M_r = 60,000$ protein was responsible for the

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*Fig. 1.* *E. coli* lysates inhibit p56<sup>Lck</sup> activity. Bacterial lysates were prepared from an overnight culture of N4830-1 cells grown at 28 °C in LB. Cells were lysed in 1/50 of their culture volume with Nonidet P-40 lysis buffer. 5 µl of a 1:4 dilution of this lysate was evaluated for p56<sup>Lck</sup> inhibitory activity by PCFIA. Cytoplasmic and periplasmic fractions were prepared from similar cultures and also assayed for inhibition. In both cases, parallel assays were performed in the presence of inhibitors of phosphatases and proteases (1 mM sodium orthovanadate, 0.8 mM sodium molybdate, 1 mM PMSF, 1.6 mg/ml leupeptin, 10 µg/ml pepstatin). *bcknd* refers to the background signal in the absence of added p56<sup>Lck</sup>. All values are reported as a percent of maximal p56<sup>Lck</sup> activity. The data shown are from one representative experiment. Error bars represent standard errors from triplicate measurements.

*Fig. 2.* Periplasmic inhibition of p56<sup>Lck</sup> autophosphorylation. Aliquots of SF9-expressed and DEAE-purified p56<sup>Lck</sup> were incubated with 1:4 dilutions of periplasmic extracts, 25 µg/ml MBP, and 0.8 mM sodium molybdate for the times indicated above each lane. Reactions were terminated with the addition of SDS sample buffer, boiled, and separated by electrophoresis. The gel was dried and exposed to x-ray film for 24 h. Lane 1, p56<sup>Lck</sup> only was incubated for 3 min. Lane 2, p56<sup>Lck</sup> only was incubated for 3 min, then periplasmic extract was added to the reaction for an additional 10 min. Lane 3, p56<sup>Lck</sup> only was incubated for 13 min. Lane 4, as for lane 2 except that molybdate was included in the reaction. Lane 5, as for lane 3 except that MBP was included in the reaction. Lanes 6 and 8, as for lane 5 except that periplasmic extract was included in the reaction. Lane 7, as for lane 6 except molybdate was included in the reaction. The 47-kDa band is an unidentified periplasmic protein that incorporates phosphate.

*Fig. 3.* Superose 12 gel filtration analysis of periplasmic inhibitor. Q-Sepharose-purified periplasmic inhibitor was analyzed on a Superose 12 gel filtration column as described under “Experimental Procedures.”

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**Table I. Summary of the Purification**

| Fraction | Activity (%) |
|----------|--------------|
| 124 kD   | 100          |
| 67 kD    | 90           |
| 440 kD   | 50           |
| background | 0          |

Note: Activity is based on the background signal (background = 0).

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**Fig. 5** shows a silver-stained SDS-PAGE gel of inhibitory fractions 22, 23, and 24 from the Mono Q column of Fig. 4. A prominent band with a molecular weight of approximately 60,000 is observed. Slices of similar SDS-PAGE gels were incubated in buffer to elute proteins, acetone-precipitated to remove SDS from protein, and the precipitate was assayed for p56<sup>Lck</sup> inhibitory activity. As shown in Fig. 6, the gel slice corresponding to the $M_r = 60,000$ protein inhibits p56<sup>Lck</sup> while eluates from the other slices do not.
Bacterial Inhibitor of Protein Kinases

**FIG. 4. Purification of the periplasmic inhibitor.** The details of each purification step can be found under “Experimental Procedures.”

| Purification step       | % Recovery from previous step | Cumulative increase in specific activity |
|-------------------------|-------------------------------|-----------------------------------------|
| Periplasmic extraction  | 100                           | 10                                      |
| Ammonium sulfate        | 95–100                        | 440                                     |
| Q-Sepharose             | 189a                          | 5,720                                   |
| Hydroxypatite           | 65                            | 15,444                                  |
| Mono Q                  | 5                             | 21,622                                  |

a Total inhibitory activity increased following this purification step.

**FIG. 5. SDS-PAGE analysis of highly purified p56\(^{ck}\) inhibitor.** Mono Q-purified inhibitor fractions 22, 23, and 24, which contain inhibitory activity, were separated by SDS-PAGE and silver-stained.

p56\(^{ck}\) inhibitory activity. Purified fractions were run on SDS-PAGE and transferred to Immobilon membrane. The region of membrane corresponding to the inhibitor was cut out, digested with trypsin, and the fragments were analyzed by mass spectrometry. 19 major peaks were identified and their molecular weights were compared to a data base of predicted tryptic fragments from bacterial proteins. 13 of the 19 peaks matched the predicted profile for the bacterial periplasmic enzyme UDP-sugar hydrolase. This enzyme was first characterized by Glaeser et al. (29) and Neu (30), and its gene (UshA) was cloned by Burns and Beacham (21). Its properties match very closely with the properties of the inhibitor including resistance to moderate heat treatment, subcellular localization (periplasm), molecular weight (60,800), requirement for divalent cations (particularly Co\(^{2+}\) or Mn\(^{2+}\), not shown), hydrophilicity, and behavior on ion exchange and other matrices.

In order to confirm that UDP-sugar hydrolase was responsible for the p56\(^{ck}\) inhibitory activity, we introduced plasmid pLA7 that contains the full gene for the enzyme into bacteria and measured enzyme and kinase inhibitory activity. Introduction of this plasmid into E. coli results in the overexpression of its enzymatic activity by 10-fold. The corresponding kinase inhibitory activity in crude lysates or partially purified fractions increases by 50-fold relative to wild-type (Fig. 7). These results are entirely consistent with the conclusion that the kinase inhibitor is in fact UDP-sugar hydrolase.

UDP-sugar hydrolase would appear to be an unusual candidate for a kinase inhibitor. However, this enzyme also possesses a potent and nonspecific 5‘-nucleotidase activity. It is thus able to hydrolyze ATP to ADP, AMP, and adenosine. One possibility is that UDP-sugar hydrolase simply depletes ATP in the kinase reaction. We measured ATP hydrolysis by partially purified inhibitor extracts under the conditions of our kinase assay (i.e. 0.3–0.5 mM ATP) and although we readily detected hydrolysis, the depletion of ATP (approximately 50%) was insufficient to account for the loss of kinase activity (Fig. 8). In fact, under the conditions of our assay, full kinase activity is still observed at 10 μM ATP. This experiment did show, however, that the primary enzymatic product was adenosine. We therefore tested for the ability of adenosine to inhibit the activity of p56\(^{ck}\). As shown in Fig. 9a, adenosine is a strong inhibitor of p56\(^{ck}\) activity with an estimated IC\(_{50}\) of 0.3 mM. Therefore, these results suggest that the production of adenosine is the mechanism by which UDP-sugar hydrolase inhibits p56\(^{ck}\).

Our observation that p56\(^{ck}\) could be inhibited by adenosine prompted us to look at inhibition by adenosine of other kinases. As shown in Fig. 9, b and c, adenosine also inhibits the activity of casein kinase and cAMP-dependent protein kinase. The estimated IC\(_{50}\) values for these two enzymes are 18 μM for casein kinase and 0.48 μM for cAMP-dependent protein kinase.

**FIG. 6. The periplasmic inhibitor can be eluted from SDS-PAGE gels.** 4-mm slices from an SDS-PAGE gel of inhibitor purified by ammonium sulfate, Q-Sepharose, and hydroxypatite were soaked overnight at room temperature in 10 mM Tris (pH 8), 0.1% SDS to passively elute the protein. BSA as carrier was added and total protein was precipitated by the addition of 5 volumes of ice-cold acetone. After 2 h at –20 °C, precipitated proteins were pelleted by centrifugation at 12,000 × g for 10 min. Pellets were resuspended in 10 mM Tris, pH 8, and then assayed for kinase inhibitory activity. The inhibitory gel slices correspond to molecular weights between 55,000 and 65,000.

UDP-hydrolase Activity Affects Invasiveness of EPEC—The ability to produce adenosine resulting in the inhibition of various protein kinases suggested that UDP-sugar hydrolase may also play a role in bacterial-host interaction. To test this possibility, we examined the effect of overexpressing UDP-sugar hydrolase in an *in vitro* model of bacterial intracellular invasion.

Enteropathogenic *E. coli* (EPEC) strains have the ability to adhere to and invade human cells; however, intracellular survival is limited and recovery of intracellular bacteria decreases over time (not shown). Monolayers of HeLa cells were infected with log phase EPEC cells containing pLA7 or the parent vector pBR322.

If the incubation period is limited to 1 h, no differences in recovery of bacteria are seen between EPEC cells containing pBR322 or pLA7 (not shown). This observation suggests that bacterial growth, adherence, and invasion are unaffected by...
the presence of UDP-sugar hydrolase. However, if the incubation period is extended to 3 h, overexpression of UDP-sugar hydrolase results in an approximate 10-fold increase in the number of bacteria recovered over a wide range of multiplicities of infection (10 to 500) (Fig. 10). These results suggest that bacteria overexpressing UDP-sugar hydrolase have an increased ability to survive once inside the HeLa cells.

Since our in vitro results suggested to us that invasive bacteria could affect signaling events once inside the cell, we performed an invasion assay and examined infected cells for alterations in phosphotyrosine content. As shown in Fig. 11, uninfected HeLa cells display a prominent phosphotyrosine-containing protein of approximately 120 kDa. 1 and 3 h after infection, the phosphotyrosine content of this band decreases in cells infected with both strains, but the decrease is greater in cells infected with EPEC overexpressing UDP-sugar hydrolase. As described earlier by Rosenshine et al. (31), infection with EPEC results in the induction of additional phosphotyrosine-containing bands of host cell origin (approximately 90 and 140 kDa). We also observe a marked reduction in phosphotyrosine content of these bands 3 h after infection with EPEC overexpressing UDP-sugar hydrolase activity. These results suggest that host cell signaling is being disrupted by invading EPEC and that UDP-sugar hydrolase overexpression enhances this effect.

DISCUSSION

p56\textsuperscript{cK}, a member of the Src family of protein-tyrosine kinases, is a T cell-specific kinase with both autophosphorylation activity and the ability to phosphorylate exogenous substrates such as myelin basic protein. We found that coinucubation of bacterial lysates with this enzyme strongly inhibits its activity. We linked the inhibitory activity to a Mr 560,000 periplasmic protein, purified it to homogeneity, and identified it as the bacterial enzyme UDP-sugar hydrolase. The inhibitor shares many biochemical properties with UDP-sugar hydrolase including its molecular weight, behavior on ion exchange and other matrices, relative resistance to heat treatment, its hydrophilicity, and its dependence on divalent cations such as Mn\textsuperscript{2+} (but not Ca\textsuperscript{2+} or Mg\textsuperscript{2+}). Overexpression of UDP-sugar hydrolase in bacteria results in a coordinate increase in 5'-nucleotidase activity as well as p56\textsuperscript{cK} inhibitory activity. Taken together, these results strongly support the identity of the kinase inhibitor as UDP-sugar hydrolase.

When first identified and characterized, the role assigned to
UDP-sugar hydrolase was as a component of the nucleotide scavenging pathway. Its ability to hydrolyze nucleotides enables the products of the reaction to enter into the bacterial cell where they can be used as nucleotide precursors, or as a carbon source. In fact, cells possessing this enzyme can grow using 5'-AMP as the sole carbon source. The role of its UDP-sugar hydrolase activity is less clear. UDP-sugars are involved in glycogen synthesis, acting as high energy intermediates. It is possible that this enzyme is also involved in scavenging these molecules.

UDP-sugar hydrolase is a potent and relatively nonspecific 5'-nucleotidase. Although it does deplete ATP present in the kinase reaction, simple loss of ATP does not seem to be the basis of its inhibitory activity. Rather, we have found that adenosine, the reaction product, is inhibitory for the protein kinases. We have also investigated the potential inhibitory activity of AMP and ADP on the kinases and find that only p56\textsuperscript{Lck} is also significantly inhibited by these molecules (IC\textsubscript{50} of 50 and 25 \mu M respectively\textsuperscript{2}). However, since adenosine is the only hydrolysis product detected under the conditions of our assay, we conclude that the production of adenosine is the mechanism by which UDP-sugar hydrolase inhibits the kinases. This result is intriguing, considering the fact that deficiencies in adenosine deaminase, the major enzyme responsible for “detoxifying” adenosine, results in a severe combined immunodeficiency syndrome, the most notable feature being severe T cell deficiency (32).

Adenosine, in addition to its metabolic role as a nucleotide precursor, also has other functions. In vivo, its main physiological function seems to be as a regulator of cardiac rhythm; however, it also has documented anti-inflammatory effects. These include inhibition of neutrophil adhesion (33), inhibition of platelet aggregation (34), and inhibition of superoxide burst by neutrophils (35, 36). Cronstein et al. (37) have shown that the anti-inflammatory effects of methotrexate are most likely mediated through a buildup of adenosine. In vitro, adenosine is a known inhibitor of phosphoinositol kinases (38), a key second messenger generating enzyme. We have shown that UDP-sugar hydrolase can inhibit a tyrosine kinase and two Ser/Thr kinases, and Kim and Matthews\textsuperscript{3} have shown that yeast histidine kinase is also inhibited. Thus, although we have characterized this enzyme primarily as an inhibitor of p56\textsuperscript{Lck}, it appears that its effects are more widespread. Therefore, overproduction of adenosine by an infectious agent may result in inhibition of a wide variety of kinases and could therefore be of general utility in compromising host function.

At the present time, we have not identified the mechanism of inhibition of protein kinases by adenosine. Preliminary data suggest that at least for p56\textsuperscript{Lck}, adenosine does not act as a competitive inhibitor for ATP.\textsuperscript{2} As well, the fact that the apparent IC\textsubscript{50} values for the kinases varies for each enzyme suggests that there may be considerable specificity. Further experiments are required to address the mechanism of inhibition and the identification of other potential targets.

The multiple kinase inhibitory activities of UDP-sugar hydrolase products coupled with its role in the metabolism of nucleotides and UDP-sugars suggested to us that this enzyme might influence bacterial intracellular infection. To test this, we investigated the effect of overexpressing UDP-sugar hydrolase in the EPEC model of \textit{in vitro} bacterial invasion. Once EPEC invade the monolayer, extensive growth does not normally occur. This assay is therefore a useful one for investigating potential factors that can affect intracellular survival. We found that overexpression significantly increases the recovery of enteropathogenic \textit{E. coli} following invasion over a wide range of multiplicities of infection, but only following a 3-h incubation period. Since no differences were seen after short incubation times, this result suggests that overexpression of UDP-sugar hydrolase increases bacterial survival once inside the HeLa cells. At present, we do not know the precise biochemical mechanism mediating this effect. However, the multiple inhibitory

\textsuperscript{2} S. A. Berger, unpublished data.

\textsuperscript{3} Y. Kim and H. R. Matthews, unpublished data.
activities of adenosine, coupled with its involvement in nucleotide scavenging, suggests that this enzyme may have an essential metabolic role, or may act as a general host "energizing" factor, possibly affecting host cell signaling. In agreement with this possibility, we found that the phosphotyrosine content of the major phosphorylated proteins in HeLa cells decreased following EPEC infection. Furthermore, this decrease was even greater in HeLa cells infected with EPEC cells overexpressing UDP-sugar hydrolase.

UDP-sugar hydrolase is widely distributed and highly conserved throughout evolution, and it is possible that other pathogens may also employ this enzyme during infectious situations. For instance, Small et al. (38) have shown that supernatants from germinated Candida albicans cultures contain adenosine which is responsible for inhibiting neutrophil function. Therefore, the induction of host cell toxicity or energy via adenosine production may represent a novel and general mechanism of pathogenicity.

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