Glutathione S-Transferase Omega 1-1 Is a Target of Cytokine Release Inhibitory Drugs and May Be Responsible for Their Effect on Interleukin-1β Posttranslational Processing*

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Stimulus-induced posttranslational processing of human monocyte interleukin-1β (IL-1β) is accompanied by major changes to the intracellular ionic environment, activation of caspase-1, and cell death. Certain diaryl-sulfonyleureas inhibit this response, and are designated cytokine release inhibitory drugs (CRIDs). CRIDs arrest activated monocytes so that caspase-1 remains inactive and plasma membrane latency is preserved. Affinity labeling with [14C]CRIDs and affinity chromatography on immobilized CRID were used in seeking potential protein targets of their action. Following treatment of intact human monocytes with an epoxide-bearing [14C]CRID, glutathione S-transferase (GST) Omega 1-1 was identified as a preferred target. Moreover, labeling of this polypeptide correlated with irreversible inhibition of ATP-induced IL-1β posttranslational processing. When extracts of human monocytic cells were chromatographed on a CRID affinity column, GST Omega 1-1 bound selectively to the affinity matrix and was eluted by soluble CRID. Recombinant GST Omega 1-1 readily incorporated [14C]CRID epoxides, but labeling was negated by co-incubation with S-substituted glutathiones or by mutagenesis of the catalytic center Cys32 to alanine. Peptide mapping by high performance liquid chromatography-mass spectrometry also demonstrated that Cys32 was the site of modification. Although S-alkylglutathiones did not arrest ATP-induced IL-1β posttranslational processing or inhibit [14C]CRID incorporation into cell-associated GST Omega 1-1, a glutathione-CRID adduct effectively demonstrated these attributes. Therefore, the ability of CRIDs to arrest stimulus-induced IL-1β posttranslational processing may be attributable to their interaction with GST Omega 1-1.

Interleukin (IL)1-1 is a proinflammatory mediator produced in abundance by lipopolysaccharide (LPS)-activated monocytes and macrophages (1). Unlike most other cytokines generated by these cells, IL-1 is not constitutively released. Rather, efficient IL-1 export requires that the cytokine-producing cell encounter an effector that initiates an unusual posttranslational processing mechanism leading to cytokine release (2–4). A separate secretion stimulus is needed because the initial IL-1 translation product lacks a signal sequence (5, 6), a recognition motif that directs nascent membrane and secretory polypeptides to the endoplasmic reticulum (7). From this location, polypeptides destined for secretion typically proceed to the cell surface via a common secretory apparatus involving the Golgi complex and small secretory vesicles (8). In the absence of the recognition marker for entry to the endoplasmic reticulum, newly synthesized IL-1 accumulates within the cytoplasmic compartment (9, 10). Proteins retained in this compartment generally are not thought to function as extracellular mediators.

Separate but related genes encode the procytokine forms of IL-1α and IL-1β (5, 6). In response to proper cellular stimuli, the 31-kDa translation products of both genes are proteolytically processed to the mature 17-kDa cytokines. In the case of IL-1α, the mature and procytokine species both are capable of binding to the IL-1 receptor complex and initiating cytokine signaling (11). In the case of IL-1β, proteolytic maturation is required to generate a receptor-competent ligand (11). Pro-IL-1β is cleaved by caspase-1 (12, 13), one of a family of intracellular cysteine proteases that initiate and/or execute apoptotic responses (14). Stimuli that promote efficient IL-1 post-translational processing in vitro include extracellular ATP (2, 15, 16), the potassium ionophore nigericin (3, 17), bacterial toxins such as Escherichia coli hemolysin (18), cytolytic T-cells (2, 19), and protegrins (20). All these agents induce membrane depolarization and cell death, and K+ efflux from the target cell appears to be a requirement for the cytokine response (15, 21). K+ efflux increasingly has been reported to be an important element of many apoptotic responses (22–24).

ATP-induced IL-1β posttranslational processing proceeds via activation of the P2X7 receptor, a ligand-gated ion channel. Immediately following ATP binding, the P2X7 receptor acts as a non-selective cation channel (25–27). However, prolonged ligation causes the channel to transition to a “porelike” conductance state (28, 29). Receptor mutagenesis has suggested that the intracellular carboxyl terminus of the receptor polypeptide is necessary for pore formation (25), but whether the pore forms as a result of a change in the receptor itself or as a result of an association with other polypeptides remains to be determined (30, 31). Changes attendant to P2X7 receptor activation include activation of various stress kinases (32, 33),

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§ The abbreviations used are: IL, interleukin; CRID, cytokine release inhibitory drug; LPS, lipopolysaccharide; GST, glutathione S-transferase; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; PMSP, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry.
IL-1 is a key contributor to many inflammatory disease processes, and both preclinical animal model as well as human stimulus-induced IL-1 payment. We recently identified a series of cytokine release inhibitors (CRIDs) that potentially and selectively disrupt stress-induced IL-1β posttranslational processing (44). These agents do not directly inhibit caspase-1, and they do not act as antagonists of the P2X7 receptor; their mechanism of action remains to be established. This paper describes studies aimed at identifying their molecular target. Our results indicate that GST Omega 1-1 may perform an important, although currently unidentified, role in ATP-induced IL-1β posttranslational processing.

EXPERIMENTAL PROCEDURES

Reagents—1-(4-Chloro-2,6-diisopropylphenyl)-3-[2-fluoro-5-oxiranyl benzenesulfonyl]urea (CP-452,759; CRID 1), 14C CRID 1, 1,1,2,3,5,6,7-hexahydro-s-indacen-4-yl)-3-[2-fluoro-5-oxiranylbenszenesulfonyl]urea (CP-456,773; CRID 2), and [14C]CRID 2 were prepared as detailed previously (45). CP-456,773 (CRID 3) was prepared by treatment of CP-452,759 (CRID 1) with iodoacetamide, and digested with trypsin (Promega chemically synthesized grade) by the method of Stone and Williams (49). Peptide mapping was performed using a standard Finnigan electrospray interface and controlled by data base search of Xcalibur.

Labeling of Intact Cells with an Epoxide CRID—Human mononuclear cells were isolated from normal volunteers as previously detailed (15). 2 × 106 mononuclear cells were seeded into each well of a 24-well dish, and monocytes were allowed to attach for 2 h at 37°C. Media and nonattached cells were then removed, and 5 ml of fresh serum-free medium (Ivitrinogen; catalog no. 12096-074) containing 0.1% penicillin/streptomycin and 100 ng/ml macrophage colony stimulating factor was added to each dish. These cultures were incubated overnight at 37°C in a humidified 5% CO2 environment and then washed with PBS (Sigma; E. coli serotype 055:B5) were introduced to achieve final concentrations of 5% and 10 ng/ml, respectively. Following an additional 3-h incubation, media were removed, the cells were washed several times with RPMI medium devoid of FBS, and then 3 ml of RPMI 1640 medium containing 20 mM Hepes, pH 6.9, and the indicated concentration of either 1H CRID 1 or [14C]CRID 2 was added, and the cultures were incubated at 37°C for 60 min. The cells subsequently were washed repeatedly with RPMI medium containing 25 mM Hepes, 5 mM sodium bicarbonate, pH 6.9, 1% FBS, 2 mM glutamine, 1% penicillin/streptomycin (Chase Medium). At this point, labeled cells in some experiments were subjected to ATP activation; 3 ml of fresh Chase Medium containing 2 mM ATP was added to each dish and the cultures were incubated for 60 min. Following this treatment, the dishes were placed on ice and ice media were removed for IL-1α assay (by ELISA). Cells, with or without ATP treatment, were extracted with 1 ml of 25 mM Hepes, pH 7.0, 150 mM NaCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1% PMSF, and 0.5% saponin for 30 min on ice. These extracts subsequently were clarified by centrifugation (45,000 rpm in a Beckman TL-A 4 rotor). Resulting supernatants were recovered as the soluble fraction and were concentrated by Centricon YM-30 (Millipore, Bedford, MA) centrifugation. The final concentrates were lyophilized overnight, then resuspended in SDS sample buffer. The saponin-insoluble microsomal pellets were solubilized directly into SDS sample buffer. All SDS-containing samples subsequently were subjected to SDS-PAGE (45,000 rpm in a Beckman TL-A 4 rotor). Resulting silver-stained samples then were fractionated by SDS-polyacrylamide gel electrophoresis, and the resulting dried gel was profiled by autoradiography and/or phosphorimagery analysis.

IL-1 Assays—To allow visualization of the mature cytokine species, a metabolic labeling approach was employed to study IL-1β posttranslational processing. For this approach, 1 × 106 mononuclear cells were added to each well of six-well multi-dishes in 2 ml of RPMI 1640 containing 25 mM Hepes, 5% FBS, 2 mM glutamine, 1% penicillin/streptomycin, pH 7.2 (Maintenance Medium). Monocytes were allowed to adhere for 2 h, after which the supernatants were discarded and the adherent cells were rinsed twice and then incubated in Maintenance Medium overnight at 37°C in a 5% CO2 environment. The cultured monocytes then were incubated with 10 ng/ml LPS for 2 h and labeled for 60 min in 1 ml of methionine-free RPMI 1640, containing 1% dialyzed FBS, 25 mM Hepes, pH 7.2, and 83 μCi/ml [35S]methionine (Amersham Biosciences; 1000 Ci/mmol). The pulse medium subsequently was discarded, the radiolabeled cells were rinsed once with 2 ml of Chase Medium, and then 1 ml of Chase Medium, with or without a test agent, was added to each well. Where indicated, ATP was added (from a 100 mM stock solution, pH 7.0) to achieve a final concentration of 2 mM. Radiolabeled monocytes were treated with ATP at 37°C for 3 h after which the medium was recovered and clarified by centrifugation; the supernatants were incubated with E. coli expressing the CRID-susceptible supernatants were harvested and added to 1 ml of Xpert X-100, 0.1 mM PMSF, 1 mM iodoacetate, 1 μg/ml pepstatin, and 1 μg/ml leupeptin by addition of concentrated stock solutions of these reagents. Adherent monocytes were solubilized by addition of 1 ml of an extraction buffer composed of 25 mM Hepes, 1% Triton X-100, 150 mM NaCl, 0.1 mM PMSF, 1 mM iodoacetate, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mg/ml overnight. Following a 30-min incubation on ice, both the media and cell extracts were clarified by centrifugation at 45,000 rpm for 30 min.

For the ELISA-based assay, 2 × 104 mononuclear cells were seeded into each well of 96-well plates in a total volume of 0.1 ml. Monocytes were allowed to adhere for 2 h, after which the supernatants were discarded and the attached cells were rinsed twice and then incubated in Maintenance Medium overnight at 37°C in a 5% CO2 environment.
These cultured monocytes were activated with 10 ng/ml LPS for 2 h after which the activation medium was removed, the cells were rinsed twice with 0.1 ml of Chase Medium, and then 0.1 ml of Chase Medium containing a test agent was added and the plate was incubated for 30 min; each test agent concentration was evaluated in triplicate wells. ATP then was introduced from a 100 mM stock solution, pH 7.0) to achieve a final concentration of 2 mM, and the plate was incubated at 37 °C for an additional 3 h. Media and 100X harvest was harvested and clarified by centrifugation, and their IL-1β content was determined by ELISA (R&D Systems, Minneapolis, MN).

Isolation of CRID-labeled THP-1 Cell Polypeptides—THP-1 cells were obtained from American Type Culture Collection (Rockville, MD) and activated with 200 ng/ml LPS for 2 h. These activated cells (5 × 10⁶ cells/ml) were labeled with 0.02 ml of [35S]CRID 1 in 1 ml PBS containing a defined tonic medium (27 mM NaCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 0.53 mM KCl, 14 mM NaHCO₃, 10 mM LPS, pH 7.0) as a suspension culture for 60 min at 37 °C, after which the cells were harvested by centrifugation, washed with phosphate-buffered saline, and suspended in 50 ml of 25 mM HEPES, 50 mM NaCl, 30 mM sodium glutamate, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 1 μg/ml leupeptin, 1 μg/ml pepstatin, pH 7.0. This suspension was subjected to nitrogen cavitation (650 p.s.i. for 15 min), and the resulting lysate was clarified by ultracentrifugation. The supernatant was recovered and subjected to an anion exchange resin (10-ml HiTrap Q column; Amersham Biosciences) equilibrated in 20 mM HEPES, 50 mM NaCl, 5 mM DTT, 1 mM EDTA, pH 7.0. A 10-ml column of the resin was employed to the column and were eluted at distinct regions of the NaCl gradient (Fig. 3). This was excised from the gel and analyzed. The 31-kDa polypeptide, however, required additional purification that ran through an anion exchange column fractionated on a Superose 12 HR 10/30 column, and the 31-kDa polypeptide was eluted from this sizing column as a single peak. When analyzed by two-dimensional gel electrophoresis and autoradiography, the Coomassie Blue staining polypeptide corresponded to a distinct Coomassie Blue staining polypeptide (data not shown). This was excised from the dried gel and analyzed. The 56-kDa enriched peak from the anion exchange column was dialyzed overnight against 20 mM sodium phosphate, 20 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 μg/ml pepstatin, 1 μg/ml leupeptin, pH 6.0, and then fractionated on a HiTrap SP column (5 ml). A linear gradient of NaCl (from 20 to 500 mM) was applied to the column, and a single radioactive peak was eluted. Fractions comprising this peak were pooled and concentrated by Centricon YM-30 centrifugation. This concentrate then was applied to the Superose 12 column. Again, a single peak of radioactivity was observed. Fractions within this peak were pooled, concentrated by Centricon YM-30 centrifugation, and fractionated by SDS-PAGE. Following autoradiography, the Coomassie Blue staining polypeptide corresponding to the radio-labeled 56-kDa species was excised for sequence analysis by digestion and LC-MS.

CRID Affinity Chromatography—Thiopropyl-Sepharose (7 g; Amersham Biosciences) initially was suspended in buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.0) containing 50 mM DTT to generate free sulfhydryl groups. The activated resin then was washed to remove DTT and was packed in a 5 ml bed of 10 mM Tris, 100 mM NaCl, 0.1% w/v sodium deoxycholate, pH 9.3, containing 120 μg of CRID 2; to allow success of the coupling to be monitored, a small amount of [14C]CRID 2 was added to the reaction mixture. After a 16-h incubation at 4 °C, the resin was recovered and washed extensively with 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 50 mM DTT, pH 7.0. Next, the resin was washed free of DTT and treated with 14 mM CRID 2 (45). The resin was resuspended in 10 mM Tris, 100 mM NaCl, 0.1% w/v sodium deoxycholate, pH 8.0, for 30 min at room temperature to cum unreacted free sulfhydryl groups. Following this step, the resin was washed extensively with 50 mM Tris, 5 mM DTT, pH 7.0. A 10-ml column of the resin was employed for the purification of THP-1 cell polypeptides; the resin was equilibrated in 25 mM HEPES, 100 mM NaCl, 5 mM DTT, 0.3% Triton X-100, 1 μg/ml pepstatin, 1 μg/ml leupeptin, pH 7.0.

4.5 × 10⁴ THP-1 cells were harvested by centrifugation and suspended in 20 ml of methionine-free RPMI medium containing 1% dialyzed FBS and 1 mCi of [35S]methionine (1000 Ci/mmol). The cells were labeled for 1 h after which they were harvested by centrifugation, washed with cold PBS to remove free [35S]methionine, and then mixed with 2.3 × 10⁹ unlabeled THP-1 cells. The combined cell pellet was suspended in 20 ml of hypotonic buffer (25 mM Hepes, 25 mM NaCl, 5 mM DTT, pH 7.0, containing Complete™ protease inhibitors (Roche Applied Science, Mannheim, Germany) and subjected to nitrogen cavitation (650 p.s.i. for 15 min). The resulting lysate was clarified by low speed centrifugation to remove unbroken cells and cell debris; the supernatant was subjected to high speed centrifugation to yield a cytosolic (soluble) fraction. This fraction was applied to the CRID affinity column; individual 2-ml fractions were collected and monitored for radioactivity.

Labeling of Recombinant GST Omega—Bacterially expressed protein was isolated as described (52). 1 μg of the recombinant protein was incubated with 2.3 × 10⁹ unlabeled THP-1 cells and CRID 2 (45). The selection of an epoxide as the reactive moiety was based on the expectation that this group reacts selectively to form covalent adducts with free sulfhydryl groups on proteins, and on the earlier demonstration that ATP-induced IL-1β posttranslational processing is disrupted by non-selective alkylating agents such as N-ethylmaleimide (38). Both of the epoxide-containing agents, CRID 1 and CRID 2, demonstrated dose-dependent inhibition of ATP-induced IL-1β posttranslational processing (Fig. 1B). CRID 1 and CRID 2 yielded IC₅₀ values in the monocyte-based assay of 350 and 250 nM, respectively (Fig. 1B). Importantly, equivalent concentrations of an epoxide-bearing non-diarylsulfonylurea test agent, 1,2-epoxy-3-(4-nitrophenoxy)propane (Fig. 1A), did not impair ATP-induced IL-1β production (Fig. 1B). Therefore, when these compounds were tested at similar concentrations, the pharmacodynamic activity demonstrated by the two epoxide-bearing diarylsulfonylurea-based compounds was not shared with a structurally distinct epoxide-containing agent.

To identify proteins for which function may be disrupted by CRID binding, radiolabeled derivatives of both CRID 1 and CRID 2 (45) were applied to cultured human monocytes. Monocytes treated with [14C]CRID 1 incorporated radioactivity into a limited number of discrete cytosolic proteins (Fig. 2A). At concentrations ≤0.3 μμ, [14C]CRID 1 labeled a single species with an apparent molecular mass of 31 kDa (Fig. 2A). As the concentration of this test agent was increased, polypeptides with apparent molecular masses of 32 and 56 kDa also became evident. In contrast, the membrane-enriched fraction derived from the CRID-treated monocytes contained no obvious radio-labeled proteins as detected by SDS-PAGE and autoradiography (data not shown).

A similar experiment was performed with [14C]CRID 2, and an identical set of cytosolic polypeptides incorporated the radiolabeled pharmacophore (Fig. 2B). Concentrations of [14C]CRID 2 ≤ 1 μμ resulted in the labeling of a single 31-kDa

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radiolabeled polypeptide. Increasing the test agent concentration to 3 and 10 μM enhanced incorporation into the 32- and 56-kDa species, whereas incorporation into the 31-kDa species appeared to reach saturation (Fig. 2B). As with cells treated with [14C]CRID 1, incorporation of CRID 2-derived radioactivity into membrane proteins was minimal (as detected by SDS-PAGE and autoradiography; data not shown).

Prior to harvesting [14C]CRID 1-treated cells in the above experiment, the test agent was removed from the medium, and the cells were washed to remove non-bound pharmacophore. They then were treated with ATP to engage IL-1β posttranslational processing. Media recovered from the ATP-treated cultures subsequently were assessed for IL-1β content by ELISA. The amount of cytokine is expressed as a percentage of that released from control cells in the absence of an effector and indicated as a function of test agent concentration. Each data point is the average of triplicate determinations.

Identification of Binding Polypeptides—Following treatment of THP-1 cells with [14C]CRID 1, radiolabeled polypeptides possessing apparent molecular masses of 31, 32, and 56 kDa were detected (Fig. 3A). As in the monocyte system, the 31-kDa...
polypeptide labeled at lower concentrations of the pharmacophore than did the 32- and 56-kDa species (Fig. 3A). At the highest test agent concentration (10 μM), the relative abundance of the 32-kDa radiolabeled species appeared higher in extracts derived from THP-1 cells than in those recovered from monocytes (compare Fig. 3A to Fig. 2A), but the labeling patterns for the two cell types were similar overall. Therefore, THP-1 cells were employed as a starting source for isolation of the binding proteins. A procedure for generating enriched preparations of the three radiolabeled polypeptides is detailed under “Experimental Procedures.” A key step in this isolation was fractionation of the [14C]CRID 1-labeled cytosol preparation on an anion exchange column that separated the three radiolabeled polypeptides (Fig. 3B). The 31-kDa protein did not bind to the anion exchange resin and was recovered in the flow-through fractions (Fig. 3B). In contrast, the 32- and 56-kDa species bound and were eluted with increasing salt; the 56-kDa species eluted earlier (peak C, Fig. 3B) than the 32-kDa species (peak D, Fig. 3B). The three proteins were further purified as described under “Experimental Procedures.”

NH2-terminal sequence analysis of the radiolabeled 31-kDa polypeptide blotted to polyvinylidene difluoride from a two-dimensional gel indicated that the protein possessed a blocked amino terminus. Following tryptic digestion and HPLC fractionation of the digest, a number of peak fractions were subjected to automated Edman sequencing and matrix-assisted laser desorption/ionization time-of-flight MS. Several peptide sequences were obtained that matched predicted tryptic peptides encoded by a deposited human cDNA (accession no. U90313), which also has a murine ortholog (accession no. U80819). The murine ortholog originally was identified as a resistance factor to radiation-induced lymphocyte apoptosis (54). The human protein, GST Omega 1-1, recently was identified as a novel member of the GST superfamily (52).

NH2-terminal sequence analysis of the 32-kDa polypeptide blotted to polyvinylidene difluoride from an SDS gel indicated that this protein was also blocked. Tryptic and Glu-C digests were prepared and fractionated by HPLC. Sequencing of multiple peptides led to identification of the 32-kDa polypeptide as CLIC1 (accession no. U93205), a protein belonging to a family of unusual ion channels (55, 56). A relationship between GST Omega 1-1 and CLIC1 previously was described based on sequence homology (57). Alignment of the amino acid sequences indicates that these two proteins share an absolute identity of 15% (Fig. 4); a region near the amino terminus (residues 24–38 of CLIC1 and 32–46 of GST Omega 1-1) is particularly well conserved. This is noteworthy, as Cys32 of GST Omega 1-1 resides at its putative active center (52).

Trypsin digestion of the 56-kDa polypeptide excised from an SDS gel yielded peptides matching those present in human carboxyl esterase as demonstrated by LC-MS and data base searching. The mass of carboxyl esterase, 60 kDa (58), is in close agreement with the apparent mass of the 56-kDa polypeptide.

Identification of CRID-binding Proteins by Affinity Chromatography—A CRID affinity matrix was generated by coupling CRID 2 to Thiopropyl-Sepharose, and soluble extracts derived from [35S]methionine-labeled THP-1 cells were applied to a column containing this matrix. Following sample application, a large peak of radioactivity was recovered in the flow-through fractions (Fig. 5A). The column subsequently was washed to achieve a constant base-line level of eluting radioactivity, after which p-nitrophenol (100 μM) was added to the buffer in an attempt to disrupt weak interactions. This resulted in a minimal increase in the amount of radioactivity eluted from the resin (Fig. 5A). Subsequent addition of CRID 3 (5 mM) to the elution buffer consistently eluted a small peak of radioactivity; allowing the column to sit overnight in the presence of CRID 3 eluted additional radioactivity (Fig. 5A, inset). In total, CRID 3 eluted 0.1–0.4% of the radioactivity applied to the column. Finally, the column was washed with 0.1% SDS; this denaturing detergent eluted a peak of radioactivity accounting for 14–16% of the total applied radioactivity (Fig. 5A). Overall, radioactivity recovered from the column ranged from 89 to 98% of the total applied.

Fractions corresponding to the various peaks of radioactivity were pooled and their polypeptide content analyzed by SDS-PAGE and autoradiography. Relative to the polypeptide pattern loaded onto the column, the flow-through peak contained a similar composition of radiolabeled polypeptides (Fig. 5B). The p-nitrophenol eluate contained one major polypeptide of 28 kDa (Fig. 5B). The CRID 3 eluate reproducibly contained two or three major polypeptide species with molecular masses of 22, 28, and 31 kDa (Fig. 5B). The 22-kDa species was not detected in one out of three experiments. Finally, the SDS eluate contained a large number of polypeptides (Fig. 5B); for the most part, these polypeptides were distinct from those eluted by CRID 3 and from those recovered in the flow-through (Fig. 5B).

Following SDS-PAGE, the three major radiolabeled proteins recovered in the CRID 3 eluate were identified by LC-MS and data base searching following trypsin digestion. Results of this analysis identified the 22-, 28-, and 31-kDa polypeptides as glyoxalase I, carbonic anhydrase (type II), and GST Omega 1-1, respectively. Based on Coomassie staining, carbonic anhydrase was the most abundant polypeptide in the CRID 3 eluate. However, as noted above, elution of this polypeptide from the
affinity column also occurred in the presence of p-nitrophenol, and prolonged washing of the column with buffer alone led to its elution (data not shown).

Recombinant GST Omega 1-1 Incorporates [14C]CRID 2—Incubation of human recombinant GST Omega 1-1 with [14C]CRID 2 resulted in a dose-dependent incorporation of radioactivity into the polypeptide (Fig. 6A). In view of the apparent conservation of an active site cysteine between GST Omega 1-1 and CLIC1 (Fig. 4) and the expectation that [14C]CRID 2 reacts with free sulfhydryl groups to mediate its covalent attachment to proteins, we reasoned that Cys32 in GST Omega 1-1 may be the site of attachment. Therefore, this site was mutated to an alanine residue, and the altered protein was treated with [14C]CRID 2. Concentrations of the radioactive pharmacophore resulting in robust incorporation of radioactivity into wild type GST Omega 1-1 yielded no discernible incorporation into the alanine-containing mutant protein (Fig. 6B). Thus, Cys32 appeared to be the site of covalent attachment.

The site of modification was studied directly by capillary LC-MS peptide mapping of tryptic digests of modified and control GST Omega 1-1. A single modification was confirmed by mass spectrometry of the intact modified protein (data not shown).
shown). A control sample, not exposed to CRID 2, was otherwise treated in the same way. LC-MS of the control digest, including data-dependent MS/MS and combined with post-run database searching using the SEQUEST algorithm, demonstrated the presence of the presumed active-site peptide FCPFAER. This peptide, with its cysteinyl residue as the S-carbamoylmethyl derivative, was eluted as a distinct peak near 42 min (marked with asterisk in Fig. 7A). Sequence coverage of the polypeptide was near 90%.

Analysis of CRID 2-modified GST Omega-1 revealed only a trace of the active-site peptide with S-carbamoylmethylcysteine (note disappearance of the 42-min peak in Fig. 7B), but demonstrated the presence of a peptide with mass 416 Da higher than that of FCPFAER, consistent with modification of Cys32 by the 416-Da epoxide CRID 2. Three peaks with the mass properties of this product were eluted in the region near 92–93 min (marked with asterisks in Fig. 7B).

MS/MS of this product (Fig. 7C) gave weak peaks corresponding to classical sequence ions for the predicted structure, but was dominated by peaks interpreted as being caused by neutral loss of the 173-Da aminodindanylid moiety plus an additional proton to preserve neutrality (173 Da in all). The intermediate stage of a hypothetical mechanism for this neutral loss is depicted in the inset to Fig. 7C. Thus, the major peak in the MS/MS spectrum at m/z = 556.6 was interpreted as arising from neutral loss of 173 Da from MH+2 of 643.4, leading to a doubly charged ion with m/z = 556.6. The classical b3 ion was detected at m/z = 667.0, but neutral loss of 173 Da from this fragment yielded a more intense ion at m/z = 493.9. The y3 ion at m/z = 619.3 was the major simple sequence ion detected, consistent with the anticipated relatively high lability of the peptide bond located to the NH2-terminal side of Pro.

To pursue more complete confirmation of the sequence, the doubly charged ion at m/z = 556.6 was subjected to MS3 using the programmable capacities of the LCQ ion trap spectrometer. The result (Fig. 7D) was interpreted in terms of classical sequence fragmentation of FCPFAER, with the Cys residue now having mass of 346.5 Da after loss of the 173-Da aminodindane. Continuous sets of congruent sequence ions from y1 to y6 and from b4 to b4 furnished conclusive evidence that Cys32 in the active site peptide FCPFAER was the site of modification by CRID 2.

Material with the mass of CRID 2-modified active-site peptide occurred in three unequal peaks with retention time near 92–93 min (Fig. 7B). All showed very similar behavior in multistage MS. The multiplicity of peaks was believed to originate with two factors, these being (i) the presence of two enantiomers of CRID 2 in the reagent and (ii) the potential for the Cys32 nucleophile to be alkylated by either of the epoxide carbon atoms.

The crystal structure of GST Omega 1-1 indicates that Cys32 is located near the boundary of the two sites in the polypeptide corresponding to the glutathione and electrophilic substrate binding pockets of prototype GST family members (52). Therefore, we asked if S-alkylglutathione analogs disrupt binding of GST Omega 1-1 to [14C]-CRID 2; a number of GSTs, including porcine GST Omega 1-1 (59), bind these glutathione adducts tightly because they mimic enzyme reaction products (60). Glutathione sulfonate (2 mM) produced little effect on the incorporation (Fig. 8A). Likewise, S-methylglutathione was inactive at all tested concentrations (up to 200 μM). As the chain length of the alkyl group increased, however, progressively greater inhibitory potencies were observed (Fig. 8A). Thus, S-ethyl-, S-butyl-, S-hexyl-, and S-octylglutathione yielded IC50 values of 350, 18, 3.0, and 0.9 μM, respectively. Further elongation of the alkyl group to 10 carbon atoms did not lead to an additional increase in potency; the IC50 for S-decylglutathione was 1.2 μM (Fig. 8A). The presence of a large aliphatic chain was not required for effective inhibitory activity as S-(p-nitrobenzyl)glutathione and S-(1-adamantyl)glutathione yielded IC50 values of 1.8 and 3.0 μM, respectively (Fig. 8A).

Several other S-substituted glutathione adducts demonstrated modest IC50 values including oxidized glutathione (350 μM), S-(p-azidophenylacetyl)glutathione (100 μM), S-(p-chlorophenacyl)glutathione (95 μM), and S-β-lactoylglutathione (200 μM). Results of these competition experiments, therefore, suggest that GST Omega 1-1 can bind to S-substituted glutathione adducts and that this binding prevents access of Cys32 to [14C]-CRID 2.

One additional glutathione adduct was synthesized and assessed in the competition assay. CRID 2 was conjugated to reduced glutathione via the epoxide group of the pharmacophore to generate an adduct of the CRID with glutathione (CRID 2-SG). This adduct blocked incorporation of [14C]-CRID 2 into GST Omega 1-1 (Fig. 8D); based on phosphorimager analysis of the gel, the adduct yielded an IC50 of 25 μM.

CRID 2-SG Blocks ATP-induced IL-1β Processing—If GST Omega 1-1 is the target responsible for the pharmacological action of CRIDs, then other agents that bind to this polypeptide may act as inhibitors of ATP-induced IL-1β posttranslational processing. To explore this possibility, several S-alkylglutathione derivatives (S-methyl-, S-hexyl-, S-octyl-, and S-decyl-glutathione) were tested individually in the monocyte cytokine production assay; all were inactive as inhibitors of ATP-induced IL-1β posttranslational processing (Fig. 9A). On the other hand, monocytes treated with CRID 2-SG demonstrated a dose-dependent inhibition in cytokine production; the IC50 in this assay was 18 μM (Fig. 9C). CRID 2 on its own demonstrated a dose-dependent response, yielding an IC50 value of 70 nM (Fig. 9C). Therefore, attachment of the glutathione tripeptide lowered CRID potency, but did not eliminate activity. Confirmation of this inhibitory effect was achieved using a metabolic assay format. LPS-activated[35S]methionine-labeled human monocytes were treated with ATP in the absence or presence of the adduct after which IL-1β released to the medium was recovered by immunoprecipitation. In the absence of the adduct, large amounts of the 17-kDa mature cytokine species were released to the medium (Fig. 9D).
CRID 2-SG adduct in the culture medium dose-dependently inhibited release of the 17-kDa species (Fig. 9D). Moreover, as observed with cells treated with a CRID (42), adduct-arrested cells did not compensate by releasing pro-IL-1β to the medium (Fig. 9D). S-Hexylglutathione (1.25 mM) did not inhibit release of 17-kDa IL-1β/H9252, confirming its inactivity observed in the ELISA format (Fig. 9A).

The inability of S-alkylglutathione analogs to inhibit the ATP response may indicate that these adducts did not access GST Omega 1-1 within intact cells; nonesterified glutathione analogs generally are not membrane-permeant (61). To assess this, monocytes were treated with [14C]CRID 2 in the absence and presence of an excess of S-hexylglutathione. In the absence of a potential effector, monocytes treated with [14C]CRID 2 readily incorporated radioactivity into 31-kDa GST Omega 1-1 (Fig. 9B). Co-treatment with 1 or 5 mM S-hexylglutathione did not reduce this incorporation (Fig. 9B). On the other hand, monocytes treated with [14C]CRID 2 in the presence of the CRID 2-SG adduct displayed reduced incorporation of the radiolabel into GST Omega 1-1; concentrations >1 mM were effective (Fig. 9B). Thus, whereas both S-hexylglutathione and CRID 2-SG blocked incorporation of [14C]CRID 2 into rGST Omega 1-1, only the latter blocked [14C]CRID 2 incorporation into cell-associated GST Omega 1-1.

**DISCUSSION**

Based on the unusual requirement for a separate secretory stimulus to promote efficient mature IL-1β export from LPS-activated human monocytes, we previously set out to identify inhibitors of this process using an intact cell assay format (44). This search led to discovery of diarylsulfonylureas as a potent and selective class of CRIDs. These agents block mature cytokine production independent of the initiating stimulus, and they arrest the process in a manner that allows the activated cells to maintain plasma membrane latency (44). This fingerprint shares features with several non-selective anion transport inhibitors, such as tenidap and ethacrynic acid, which also arrest stimulus-induced IL-1β production (62); the molecular mechanism by which these agents disrupt the cytokine process is not known. In an attempt to elucidate the mechanism by which diarylsulfonylureas disrupt the cellular process, we employed a multifaceted approach. Results of our search identify GST Omega 1-1 as a diarylsulfonylurea-binding protein and suggest that this interaction is responsible for inhibition of ATP-induced IL-1β posttranslational processing. Evidence supporting these conclusions include demonstrations that: 1) [14C]-labeled epoxide-containing diarylsulfonylureas bind irreversibly to GST Omega 1-1 and this binding correlates with
inhibition of cytokine production, 2) GST Omega 1-1 binds reversibly to a diarylsulfonylurea affinity column, and 3) a CRID-glutathione adduct inhibits ATP-induced IL-1β post-translational processing and interacts with GST Omega 1-1 within intact monocytes.

The radiolabeled epoxide-containing diarylsulfonylureas CRID 1 and CRID 2 both covalently labeled three monocysteoluble polypeptides corresponding to GST Omega 1-1 (31 kDa), CLIC1 (32 kDa), and carboxylesterase (56 kDa). Alveolar macrophages are known to express and secrete carboxylesterase (58); the exact role of this enzyme in the macrophage is not clear. Relative to concentrations of the test agents required to irreversibly block ATP-induced IL-1β processing, carboxylesterase labeling was observed only at concentrations in excess of those required to inhibit the cytokine response. Moreover, when tested as a direct inhibitor of purified carboxylesterase, 50 μM CRID 2 yielded a modest inhibitory effect (55%; data not shown). Therefore, carboxylesterase is not considered a good candidate to account for the CRID pharmacodynamic effect.

GST Omega 1-1 and CLIC1 are related polypeptides sharing 15% sequence identity. Moreover, the crystal structure of GST Omega 1-1 predicts the presence of an active site cysteine (Cys32) (52), and this residue is conserved in CLIC1. Importantly, substitution of alanine for cysteine (52) in GST Omega 1-1 dramatically reduced the ability of the recombinant protein to incorporate [14C]CRID 2, and LC-MS peptide analysis indicated that this cysteine residue is responsible for nucleophilic attack of the pharmacophore-associated epoxide group. Incorporation into GST Omega 1-1 occurred at lower concentrations of the radiolabeled pharmacophores than did labeling of CLIC1, suggesting that the former has a higher binding affinity for the diarylsulfonylureas or that CLIC1 has an inherently lower nucleophilic capacity. Recent resolution of the x-ray crystal structure of CLIC1 indicates that this polypeptide not only shares sequence similarity to GST Omega 1-1 but also structural attributes (63). Therefore, the ability of [14C]CRID 2 to label both of these polypeptides may reflect conservation of structure and/or function.

Concentrations of [14C]CRID 2 required to label cell-associated GST Omega 1-1 correlated with the extent of inhibition of IL-1β posttranslational processing. It is noteworthy that cell-associated GST Omega 1-1 incorporated the epoxide-bearing pharmacophores. In the previous x-ray crystallography study, Cys32 of recombinant GST-Omega was found to readily form a mixed disulfide bond with glutathione (52). Although intracellular concentrations of reduced glutathione are quite high (64), concentrations of the oxidized species generally are much lower and this latter species is likely necessary for formation of the mixed disulfide with GST Omega 1-1. Thus, in the context of a typical intracellular reducing environment, cell-associated GST Omega 1-1 appears to exist with the sulphydryl side chain of Cys32 unmodified.

A concern with the affinity labeling approach is that incorporation of the epoxide-bearing pharmacophore by a protein may occur simply as a result of a highly nucleophilic sulphydryl group. In view of nucleophilic cysteines being associated with many cellular proteins, however, this explanation does not appear consistent with the observed selectivity of the labeling profile. As an alternative approach, an affinity matrix was generated in which the epoxide group of CRID 2 was conjugated to an insoluble support, making it unavailable to react with protein-associated nucleophiles. Using this resin, glyoxalase I, carbonic anhydrase, and GST Omega 1-1 demonstrated CRID-dependent binding interactions. Enzymatic activity of...
recombinant glyoxalase I was inhibited by 45% in the presence of 50 μM CRID 2 (data not shown), a level not considered sufficient to account for the submicromolar CRID-like effect of this compound. Carbonic anhydrase (type II) also bound to the CRID affinity column, but elution of this enzyme was not dependent on a competing diarylsulfonylurea. Rather, bound carbonic anhydrase was eluted in the absence of a soluble competitor, although the presence of p-nitrophenol or CRID 3 enhanced its rate of elution. In an enzyme activity assay using commercial human carbonic anhydrase type II, 50 μM CRID 2 neither activated nor inhibited activity. Moreover, a known inhibitor of carbonic anhydrase, chlorothiazide, did not block ATP-induced IL-1β posttranslational processing (data not shown). Thus, the interaction of CRIDs with carbonic anhydrase is not considered responsible for the CRID pharmacological effect.

Of the three polypeptides recovered from the affinity column, only GST Omega 1-1 also was identified by affinity labeling. As noted above, binding to the affinity matrix is not dependent on formation of a covalent bond; the epoxide group of the affinity ligand tethers the pharmacophore to the resin and thus is not available to interact with Cys32 on GST Omega 1-1. Therefore, GST Omega 1-1 recognizes the diarylsulfonylurea pharmacophore independent of the presence of the epoxide.

As noted earlier, the x-ray crystal structure of GST Omega 1-1 predicts the presence of a glutathione-binding site similar to that found in prototypical GST family members (52). Based on this, a number of S-substituted glutathione adducts were assessed as inhibitors of [14C]CRID 2 incorporation into recombinant GST Omega 1-1. Structurally distinct adducts demonstrated dose-dependent reductions in radiolabel incorporation, with S-octylglutathione being the most potent inhibitor tested. This inhibition is consistent with [14C]CRID 2 binding to Cys32, which is situated adjacent to the glutathione binding pocket of the enzyme (52). On the assumption that binding to GST Omega 1-1 is responsible for the CRID effect, we asked whether the S-substituted glutathione adducts blocked ATP-induced IL-1β posttranslational processing. Several tested adducts did not inhibit the ATP response, and they did not block incorporation of [14C]CRID 2 into cell-associated GST Omega 1-1. Because the glutathione adducts block incorporation into recombinant but not cell-associated protein, we assume that the plasma membrane poses a barrier that does not allow their access to GST Omega 1-1. Significant, however, the CRID 2-SG adduct did inhibit ATP-induced IL-1β posttranslational processing and did reduce incorporation of [14C]CRID 2 into monocyte-associated GST Omega 1-1; attributes that selectively allow this adduct to penetrate the plasma membrane are unknown. The ability of the CRID 2-SG adduct to retain CRID activity despite a large structural change in the pharmacophore attendant to the presence of the tripeptide is remarkable and completely consistent with GST Omega 1-1 being involved in the cellular response mechanism. In this regard, ethacrynic acid, a compound structurally distinct from diarylsulfonylureas, also effectively inhibits ATP-induced IL-1β posttranslational processing (62). In addition to inhibiting ion transport processes, ethacrynic acid is known to inhibit members of the GST superfamily (65), and its ability to disrupt the IL-1 response further suggests a GST involvement in the cellular process.

GST Omega 1-1 is distinguished from other GST family members by the presence of an extended amino terminus and
an active center cysteine residue; most GSTs possess an active center tyrosine or threonine residue (60, 66). Absence of a side-chain hydroxyl group results in loss of prototypical glutathione conjugating activity (67); this group has been shown by x-ray crystallography to stabilize the thiolate anion of glutathione (60, 66). The active center of GST Omega appears more comparable with that expected for a glutaredoxin-type of activity than for a GST (52, 68). Indeed, human GST Omega 1-1 demonstrates modest glutathione-dependent thiol transferase activity (52). Other activities have been reported for GST Omega 1-1. For example, the rat enzyme is reported to be a dehydroascorbate reductase (69), the human enzyme is reported to modulate activity of the ryanodine receptor (57), and the murine enzyme (p28) is reported to be a glutathione-binding stress protein (54). The murine protein originally was identified on the basis that it was up-regulated in a population of T-cells resistant to radiation-induced apoptosis (54). Interestingly, this protein changed its subcellular localization in response to heat stress (54). In line with the stress connection, human GST Omega 1-1 demonstrates stronger sequence homologies with a number of plant and lower organism GSTs than to prototype mammalian GSTs, and many of these enzymes are induced in response to stress stimuli such as salt and oxygen (70–72). The actual enzymatic function of GST Omega 1-1 remains to be elucidated.

Is GST Omega 1-1 likely to function in the context of stimulus-coupled IL-1β posttranslational processing? Based on reported activities of GST Omega-1, two types of functional contributions can be envisioned. The first is based on its similarity to CLIC1. Despite our recovery of CLIC1 from the soluble fraction of THP-1 cells, this polypeptide is reported to serve as a chloride channel both in its recombinant state after incorporation into lipid vesicles and in its native state within cells (55, 56). GST Omega-1 itself has not been reported to possess ion channel activity, but CLIC1 and GST Omega-1 are related polypeptides; they share sequence homology, possess similar x-ray crystal structures, and are labeled by epoxide-bearing CRIDs. We assume, therefore, that these two polypeptides share functional attributes. ATP-induced IL-1 posttranslational processing is accompanied by dramatic changes in ionic homeostasis, and yield of extracellular cytokine is greatly affected by changes to the ionic composition of the medium. For example, increasing extracellular K+ (15), removal of extracellular Na+ (73), or replacement of extracellular Cl− with chaotropic anions inhibits the cellular response (74). Thus, CLIC1 and GST Omega-1 may function to facilitate an important ionic flux. In this regard, Escherichia coli contains a glutathione-gated potassium efflux system that operates in response to stress and redox state (75, 76) and perhaps GST Omega-1 is a component of this type of mechanism.

An alternate function for GST Omega-1 is suggested based on similarity between its active center and that of glutaredoxin (52). Glutaredoxin possesses both dehydroascorbate reductase and thioltransferase activities (77, 78). Effectors that induce monocyte/macrophage IL-1 processing in vitro promote dramatic changes to the intracellular environment and ultimately cause the responding cell to die. The best-studied effector, ATP, works via the P2X7 receptor. When ligated, this receptor can initiate activation of caspases, phospholipases, and stress kinases (32–36); all of these activities signal that the responding cell is engaged in a stress response. Moreover, a recent study using rat microglial cells noted that P2X7 receptor activation promotes a burst of H2O2 generation (79), suggesting that receptor ligation also alters intracellular redox state. During conditions of oxidative stress, increased levels of oxidized glutathione and glutathionylation of select cellular polypeptides can be observed (80–84). This reversible posttranslational modification can lead to altered protein/enzyme function, suggesting a new type of regulatory mechanism. For example, tyrosine hydroxylase becomes glutathionylated and inactivated when PC12 cells are treated with the oxidant diamide (85). Removal of the glutathione moiety restores enzyme activity, and glutaredoxin catalyzes this thioltransferase type of reaction (85, 86). Caspases, as a family of cysteine proteases, represent prime targets for glutathionylation at their active center thioles. Indeed, purified caspase-3 forms a mixed disulfide and is inactivated following treatment with oxidized glutathione (87). Interestingly, activation of caspase-8 via the death-inducing signaling complex in FAS-induced lymphocytes is dependent on the presence of glutathione (88). The glutathione-dependent step has not been determined, but appears to occur after assembly of the protein complex (88). The mechanism of caspase-1 activation is not well understood, but like caspase-8, appears to involve a large protein assembly termed the inflammasome (89). Perhaps an ATP-induced change in the intracellular redox state promotes formation of glutathione-protein mixed disulfides leading to inactivation of cellular polypeptides such as caspase-1 or a member of the inflammasome, function of which is critical to the cellular response. To maintain activity of critical sulfhydryl-containing enzymes, GST Omega-1 may act in concert with glutaredoxin to reduce glutathione-protein mixed disulfides and preserve catalytic function. Although recombinant GST Omega-1 demonstrates very low activity as a thioltransferase when assessed with the artificial substrate hydroxyethyl disulfide (52), the possibility that this type of activity could be achieved with a protein-linked disulfide remains to be tested. As noted above, the Omega class of GSTs is evolutionarily conserved in plants. The Arabidopsis thaliana genome, for example, contains at least five separate genes encoding GST family members possessing the conserved CPF active center motif (90). Although biological function of the plant enzymes also remains to be elucidated, conservation within both the plant and animal kingdoms suggests that this family of enzymes serves an important function worthy of further investigation.

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Glutathione S-Transferase Omega 1-1 Is a Target of Cytokine Release Inhibitory Drugs and May Be Responsible for Their Effect on Interleukin-1 β Posttranslational Processing

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