Accumulation of Diacylglycerol in the Chlamydia Inclusion Vacuole

POSSIBLE ROLE IN THE INHIBITION OF HOST CELL APOPTOSIS*

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Intracellular pathogens have developed strategies to survive for extended periods inside their host cells. These include avoidance of host microbicidal effectors, often by sequestration in a protected subcompartment of the host cell. In some cases, the parasites exert also an anti-apoptotic effect that prolongs the life of the infected host cell. Chlamydia utilizes both strategies, but the underlying molecular mechanisms are incompletely understood. Comparatively, little is known regarding the effects that Chlamydia exerts on the metabolism and distribution of the host cell lipids. The expression of fluorescently tagged C1 domains revealed that diacylglycerol is greatly accumulated in the immediate vicinity of Chlamydia inclusion vacuoles. The concentrated diacylglycerol recruits protein kinase Cδ (PKCδ), a pro-apoptotic effector, to the immediate vicinity of the vacuole. PKCδ normally exerts its pro-apoptotic effects at the mitochondria and in the nucleus. We speculate that Chlamydia antagonizes the pro-apoptotic effect of PKCδ by sequestering the enzyme on the inclusion vacuole away from its conventional target sites. Accordingly, we found that the ectopic expression of a catalytic fragment of PKCδ that cannot be recruited by the vacuole, because it lacks a functional C1 domain, overcame the anti-apoptotic effect of the bacteria. The scavenging of pro-apoptotic factors may provide a novel mechanism whereby pathogens promote their own survival by extending the life of the host cells they infect.

Chlamydia trachomatis, an obligate intracellular pathogen, propagates within large inclusion vacuoles in host cells. While excluded in the inclusion vacuole, Chlamydia not only evades immune surveillance but also avoids exposure to the microbicidal contents of the host cell lysosomes. In addition, Chlamydia manages to survive for extended periods, at least in part, by exerting an anti-apoptotic effect on host cells (1, 2). As a result, chronic infection by Chlamydia is a common occurrence, causing sustained inflammation of the genitourinary tract, eye, joints, and/or blood vessels. Little is known regarding the mechanism whereby Chlamydia antagonizes apoptosis of infected host cells. It has been demonstrated that the release of cytochrome c from mitochondria and the subsequent activation of an apoptotic protease, caspase 3, are impaired in infected cells (1, 2). Very recently, the destruction of the proapoptotic BH3-only proteins Bim/Bod, Puma, and Bad during infection was shown to contribute to the anti-apoptotic effect (3). However, the events that precede and trigger these abnormalities are not clear.

Members of the protein kinase C (PKC) family function as upstream regulators of the apoptotic pathway and are potential targets for alteration by Chlamydia. PKCs, a lipid-dependent family of serine/threonine kinases, are grouped into three subfamilies based on their structure. Classical (α, β, γ) and novel (η, ε, δ, θ) PKCs contain a diacylglycerol (DAG)-binding domain, known as the C1 cassette, which is required for optimal catalytic activity but is missing in the atypical (ζ, ξ) isotypes. Importantly, PKCδ is a pro-apoptotic regulator. When activated, PKCδ is cleaved, releasing a catalytically active fragment that translocates to the mitochondria, where it promotes the release of cytochrome c and subsequent activation of the apoptotic pathway (4–7). PKCδ also promotes inactivation of DNA-dependent protein kinases responsible for the repair of DNA double-stranded breaks (8) and phosphorylates lamin, causing disassembly of the nuclear lamina (9).

Because it is situated upstream of cytochrome c release in the apoptotic pathway, we considered whether PKCδ is involved in mediating the anti-apoptotic effect of Chlamydia. This study demonstrates that Chlamydia sequesters the majority of the enzyme on the inclusion vacuole. Recruitment of PKCδ by the chlamydial vacuole requires DAG and is mediated by the C1 domain of the kinase. We propose that, by diverting PKCδ to the vacuole away from the cellular targets that trigger apoptosis, Chlamydia may prevent the host cells from undergoing apoptotic suicide.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Dulbecco’s modified Eagle’s medium, Ham’s F-12 medium, and fetal bovine serum were from Wisent (St.

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Bruno, Quebec, Canada). Nutridoma-SP medium was from Roche Applied Science. Etoposide, rottlerin, propranolol, sodium oleate, and PMA were purchased from Sigma. Fumonisins B1 was from Biomol (Plymouth Meeting, PA). Anti-GM130 was from Transduction Laboratories (Lexington, KY). Cy3-conjugated anti-mouse antibodies were from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA).

Cell Culture and Transfection—HeLa cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cells were grown at 37 °C in 95% air and 5% carbon dioxide. Transient transfection of HeLa cells with pm-GFP, full-length PKCα, β, δ, ϵ, -GFP, the C1 domain of PKCδ-GFP, and PKC-CF6 or CF6KD was performed utilizing FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Cells were used after 24 h of transfection.

LY-B is a Chinese hamster ovary cell mutant defective in serine palmitoyltransferase, the enzyme that catalyzes the first step of sphingolipid biosynthesis (10). These cells were grown in Ham’s F-12 medium with 10% fetal bovine serum at 33 °C. To inhibit sphingolipid synthesis, the cells were grown in a sphingolipid-deficient Nutridoma-BO medium (Ham’s F-12 medium containing 1% (v/v) Nutridoma-SP and 10 μM sodium oleate complexed to albumin).

The plasmids for expression of the catalytically active (CF6) and inactive (CF6KD) fragments of PKCδ tagged with GFP were constructed as described earlier (11). GFP-tagged forms of PKCα, β, δ, ϵ and the excised C1 domain of PKCδ were kindly provided by Dr. T. Meyer (Stanford University).

Bacterial Culture and Infection Protocol—C. trachomatis serotype L2 elementary bodies were grown and maintained as frozen stocks and kept at −70 °C. For infection experiments, HeLa cells were grown on 25-mm coverslips placed within individual wells of a 6-well plate. Each well was infected by the addition of 0.5 ml of Chlamydia stock (~10⁶ elementary bodies/ml). The plates were centrifuged at 2000 RPM for 30 min to sediment the bacteria on the cells, and infected cells were then incubated for the indicated time at 37 °C.

Apoptosis Determinations—Apoptosis was induced by the addition of etoposide (10 μg/ml) followed by incubation at 37 °C for the specified time period. Apoptosis was assessed after staining the cells with DAPI (Molecular Probes, Eugene, OR) or Hoechst 33342 dye (ATCC) by monitoring nuclear morphology. Images were obtained using a Leica DMIRB fluorescence microscope, acquiring images digitally utilizing a cooled charge-couple device camera (MicroMax, Princeton Instruments, Trenton, NJ) controlled by the Winview software. ×100 oil immersion objectives were used alongside appropriate filter sets. All of the cells and inclusion bodies were identified under DIC optics. Confocal microscopy was performed with a Zeiss LSM 510 laser-scanning confocal microscope.

RESULTS AND DISCUSSION

To determine whether PKCδ mediates one of the anti-apoptotic effects exerted by Chlamydia, we analyzed nuclear condensation in HeLa cells stained with DAPI. Nuclear condensation and DNA fragmentation are hallmarks of apoptosis (12). As reported by others (13–16), we verified the effectiveness of etoposide as an inducer of apoptosis. Nearly 40% cells treated with etoposide (10 μg/ml) for 24 h showed distinct signs of apoptosis (Fig. 1, A and E). This figure is probably an underestimate, because the density of cells remaining on the coverslips was decreased compared with untreated controls, probably by detachment of cells in advanced states of apoptosis. The involvement of PKCδ as one of the inducers of apoptosis by etoposide was suggested by the preventive effects of rottlerin. This potent and reportedly specific inhibitor of PKCα, δ, and ϵ (17) decreased the incidence of nuclear condensation by >75% and prevented the detachment of cells induced by etoposide (Fig. 1, B and E). A remarkably similar phenotype was observed when the cells were infected by Chlamydia 10 h before the addition of etoposide (Fig. 1, D and E). Infection by Chlamydia in the absence of etoposide had no discernible effect on either cell survival or nuclear condensation (Fig. 1C). These observations suggest that Chlamydia might impair cellular apoptosis in part through alteration of PKCδ.

The preceding observations warranted more detailed analysis of PKCs in Chlamydia-infected cells. Because other isoforms have effects on apoptosis (PKCα and the atypical PKCs have anti-apoptotic effects (18–20)), we compared the distribution of different PKC classes in control and infected cells. HeLa cells were transiently transfected with GFP-tagged constructs of classical (α, β, δ), novel (ϵ), or atypical (ζ) isoforms of PKC. As reported earlier, in otherwise unstimulated cells, PKCα was homogeneously distributed in the cytoplasm (Fig. 2A). This distribution persisted in Chlamydia-infected cells where the formation of inclusion vacuoles was visible as zones of exclusion of the fluorescent kinase (Fig. 2, B–C). Similar results were obtained when analyzing the distribution of the atypical PKCζ (Fig. 2, G–I). In untreated cells, PKCδ was also found in

Fig. 1. Chlamydia and PKCδ inhibitor, rottlerin, prevent apoptosis. HeLa cells induced to undergo apoptosis by the addition of etoposide (10 μg/ml). After indicated time periods, cells were fixed, stained with DAPI, and examined for nuclear condensation and fragmentation. Where indicated, cells were infected with Chlamydia 10 h prior to the addition of etoposide. The PKCδ inhibitor rottlerin (5 μM) was added where specified. A, cells treated for 24 h with etoposide. B, cells treated with etoposide and rottlerin. C, cells infected with Chlamydia not treated with etoposide. D, cells treated with etoposide following infection by Chlamydia. Images are representative of three experiments of each type. E, quantification of extent of apoptosis (as percent of remaining cells) following treatment with etoposide ± rottlerin or Chlamydia infection. Data are means ± S.E. of three experiments. A minimum of 100 cells was counted for each condition/experiment. Bar = 5 μm.
the cytosol; however, a considerable fraction was associated with a juxtanuclear organelle, characterized earlier as the Golgi complex (21). The distinctive behavior of classical and novel PKC isoforms, despite their common ability to bind DAG, has been attributed to their differential ability to associate with other ligands such as arachidonic acid, ceramide, phosphatidylserine, or PKC-adaptor proteins including \( \beta \)-coatamer proteins (22–24). Remarkably, the vast majority of the novel kinase relocalized to the inclusion vacuole and/or its immediate vicinity when cells were infected by \textit{Chlamydia} (Fig. 2, D–F).

The selective redistribution of PKC\( \delta \) in infected cells suggests that prolonged cell survival may be due to neutralization of its pro-apoptotic effect and not to activation of the anti-apoptotic isoforms (PKC\( \alpha \) and PKC\( \zeta \)). Sequestration of PKC\( \delta \) to the inclusion vacuole or its immediate vicinity and away from its mitochondrial and nuclear targets might suffice to preclude its pro-apoptotic effect.

We proceeded to analyze the mechanism whereby PKC\( \delta \) relocalizes to the inclusion vacuole. The C1 domain of PKC\( \delta \) is required for its membrane association, apparently by interac-
tion with lipid moieties such as DAG or sphingolipids, both of which are present in the Golgi (25, 26). Although the components of the chlamydial inclusion membrane have not been fully characterized, it probably contains lipids found in the Golgi since traffic between this organelle and the inclusion membrane was reported (27–29). Therefore, we examined the contribution of the C1 domain in the recruitment of the kinase to chlamydial vacuoles. The C1 cassette excised from PKCδ was fused to GFP and expressed in HeLa cells. As illustrated in Fig. 2J, the chimeric C1 domain localized in intact cells in a manner that resembled the distribution of the full-length kinase. More importantly, the excised C1 domain was also avidly sequestered by the inclusion vacuole following *Chlamydia* infection (Fig. 2, K–L).

The ligands that direct the C1 domain of PKCδ to the inclusion vacuole were investigated next. C1 domains recognize DAG and sphingolipids in the context of ancillary protein moieties. To ensure that interaction with the lipids was the determinant factor, we performed competition experiments. In addition to binding endogenous lipids, C1 domains interact strongly with phorbol esters (30, 31), which are tumor promoters. As illustrated in Fig. 3A, inset, the C1 domain is effectively displaced upon the addition of PMA from the Golgi and cytosol to the plasma membrane where the tumor promoter partitions preferentially.

There are two main cellular sources of DAG: the hydrolysis of phospholipids by phospholipase C and the dephosphorylation of phosphatidic acid. The latter pathway, which is thought to be the major source of DAG for the Golgi complex (32), can be impaired by propranolol, a potent blocker of phosphatidic acid phosphatase. Consistent with this finding, the addition of propranolol to the HeLa cells displaced the C1 domain from the Golgi of uninfected cells (Fig. 3B) without affecting the structure or distribution of the Golgi complex (e.g. Fig. 3B, right inset). When both propranolol and PMA were present, the C1 domain was targeted to the plasma membrane (Fig. 3B, left inset), implying that propranolol affected DAG generation and not the behavior of the probe. More importantly, the phosphatase inhibitor similarly induced the loss of the C1 domain from *Chlamydia* inclusion vacuoles (Fig. 3D). Similar results were obtained using the full-length kinase (not illustrated). These observations confirm that lipid binding is indeed the determinant of C1 and PKCδ association with the *Chlamydia* vacuoles and point to DAG and not sphingolipids as the predominant ligand. The latter notion was further substantiated using LY-B cells, a Chinese hamster ovary line with a mutation in serine palmitoyltransferase that catalyzes the condensation of palmitoyl-CoA and 1-serine to produce 3-ketodihydrosphingosine, the initial step in the *de novo* synthesis of sphingolipids (10).

Under conditions where the LY-B cells fail to synthesize ceramide derivatives, the C1 domain nevertheless remained associated with the Golgi complex of uninfected cells (Fig. 3E). Following infection, the C1 cassette translocated to the inclusion vacuoles, despite the depletion of ceramide (Fig. 3F). In accordance with these observations, we also found that the distribution of the C1 domain in both uninfected and infected cells was not altered by pretreatment with fumonisin (Fig. 3, G–H), a potent inhibitor of dihydrosphingosine-N-acyltransferase, which is responsible for the production of dihydroceramide, an upstream precursor of ceramide synthesis (33). Similar results were obtained when we tested the effects of the ceramide antagonists on the localization of the full-length PKCδ (results not shown). Jointly, these results suggest that DAG, as opposed to sphingolipids, is the primary determinant of PKCδ recruitment to the *Chlamydia* inclusion vacuole.

The preceding observations implicate PKCδ in the anti-ap-optotic effect exerted by *Chlamydia*. The bacteria may prevent cell death by recruiting the kinase to the inclusion vacuole, diverting it away from sites such as mitochondria and the
nucleus where the apoptotic effect is normally exerted. If this hypothesis is correct, interference with the ability of PKCδ to bind to the vacuole would favor the apoptosis of cells infected with *Chlamydia*. Because DAG was established to direct the kinase to the vacuole, this could in principle be accomplished by infection of cells in medium containing propranolol. However, we found that incubation with propranolol for the extended period required for assessment of apoptosis was toxic to the cells. As an alternative approach, we used a truncated mutant of PKCδ that lacks the targeting C1 domain. Deletion of the N-terminal 325 residues of PKCδ yields a catalytically active fragment (CFδ) that is no longer dependent on DAG for its targeting or activity (11, 34), as confirmed by its inability to accumulate in the Golgi complex (Fig. 4A). In agreement with earlier results (11, 15), we found that expression of a GFP-tagged CFδ in HeLa cells effectively induced apoptosis. Nearly 70% of cells transfected with this chimeric construct underwent programmed cell death after 24 h, visualized as nuclear condensation and fragmentation (Fig. 4). Apoptosis was caused by the catalytic activity of the fragment, because transfection with a kinase-dead form of the fragment (CFδKD) had no significant effect on cell death (Fig. 4J). The effect of CFδ was next tested on cells infected with *Chlamydia*. It was notable that, unlike the full-length kinase, the CFδ was not recruited preferentially to the vacuole but instead retained access to other cellular compartments. The catalytic fragment also promoted apoptosis in cells infected 6 h after transfection, a time chosen to allow the expression of the construct. The extent of apoptosis was virtually identical to that seen in uninfected cells and much greater than that noted in CFδKD transfected and in untransfected cells (Fig. 4, H–J).

It was conceivable that, by transfacing the cells before infection, they had entered an irreversibly apoptotic program before the bacteria could exert its anti-apoptotic effects. To analyze this possibility, the experiments were also performed in the reverse order, infecting before transfection. Similar results were obtained whether CFδ transfection was performed 6 h before or 6 h after bacterial infection (Fig. 4J, cf. solid and open bars). These findings indicate that, although *Chlamydia* infection can overcome the apoptosis induced by activation of the full-length endogenous PKCδ, it is unable to prevent the effect of the C1-deficient CFδ. Therefore, we conclude that the ability of the *Chlamydia* vacuole to sequester PKCδ is essential for its anti-apoptotic action.

In summary, our findings suggest that *Chlamydia* may prevent apoptosis of infected host cells, at least in part, by disabling the pro-apoptotic effect of PKCδ. Impairment of the apoptotic action of the kinase appears to be due to its sequestration onto the vacuole and away from the normal mitochondrial and nuclear targets that initiate cell death and not due to the direct impairment of its catalytic activity. This was verified by the addition of PMA, which not only displaced the kinase from the vacuole but induced its activation, measured by autophosphorylation of Ser-645 (not illustrated). The targeting of C1 domains to the vacuole and its sensitivity to inhibitors of phosphatidate phosphatase, but not to ceramide antagonists, suggest that the membrane limiting the *Chlamydia* inclusion is rich in DAG. Unfortunately, it is currently impossible to isolate vacuolar membranes of sufficient purity and quantity to verify this assumption by direct biochemical means. Regardless of the mechanism of recruitment, it is clear that these bacteria have the unique ability to induce a gross yet selective redistribution of pro-apoptotic kinases while leaving the anti-apoptotic isoforms unaltered and that bypassing such recruitment by expression of the CFδ overcomes the pro-survival effect of *Chlamydia*. Thus, the scavenging of pro-apoptotic factors emerges as a possible novel mechanism to delay programmed cell death, which can be used by pathogens to extend the life of the host cells.

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