Activation-induced Aggregation and Processing of the Human Fas Antigen

DETECTION WITH CYTOPLASMIC DOMAIN-SPECIFIC ANTIBODIES

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Fas (APO1/CD95) is a type 1 transmembrane protein critically involved in receptor-mediated apoptosis. Previous studies have shown that Fas exists in monomeric form in resting cells and aggregates upon cross-linking to form a complex that serves to recruit additional signaling molecules to the cell membrane. To study the molecular fate of the Fas antigen following receptor activation, a monoclonal antibody specific for the cell death domain of Fas has been generated. This monoclonal antibody (3D5) could be used in Western blot analysis using total cell lysates to identify different forms of Fas antigens without immunoprecipitation. High molecular mass (>200 kDa), SDS- and β-mercaptoethanol-resistant Fas aggregates were formed immediately following receptor cross-linking, and a 97-kDa band (p97) was detected about 2 h later. p97 could be detected by antibodies against either the death domain or the C terminus. However, p97 could not be precipitated by antiextracellular domain antibodies. Thus, p97 most likely represents a processed form of the high molecular weight Fas aggregates. p97 generation followed a similar time course as CPP32 activation and poly(ADP-ribose) polymerase cleavage, it could not be inhibited by cysteine protease, calpain, or proteasome inhibitors.

Molecular weight complex upon receptor ligation (2). Mutational analysis has revealed 70 amino acid residues in the cytoplasmic domain that are critical for cell death signaling (3). This cytoplasmic domain, also found in TNFR1, is called the death domain because of its role in cell death induction (4). Recent studies have revealed that death domains can homodimerize (5) and serve to recruit signaling molecules to Fas. Upon cross-linking of the Fas antigen, FADD/MORT1, another death domain-containing protein, is recruited to the cell membrane (6, 7). FADD/MORT1 then recruits FLICE/MACH, a novel protein with a cysteine protease domain, to the complex (8, 9). Thus, in principle, Fas signaling could be accomplished by sequential recruitment of FADD/MORT1 and FLICE/MACH to the receptor complex, which may lead to FLICE/MACH activation and triggering of the subsequent protease cascade (10).

To examine the biochemistry of Fas signaling in more detail, a monoclonal antibody against the cytoplasmic domain of Fas was prepared. This monoclonal antibody (3D5) can be used in Western blot analysis using total cell lysates, allowing for examination of different forms of Fas antigens without immunoprecipitation. Furthermore, 3D5 allows for detection of Fas fragments that lack the extracellular domain. Here, we show that a 97-kDa band (p97) was detected about 2 h after formation of high molecular weight Fas aggregates. p97 cannot be precipitated by anti-extracellular domain antibody but can be detected with anti-C terminus antibody. It most likely represents a processed form of Fas that lacks the extracellular domain. Although p97 generation parallels CPP32 activation and PARP cleavage (11–13), it cannot be inhibited by cysteine protease inhibitors. Furthermore, p97 generation cannot be inhibited by calpain and proteasome inhibitors.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Burkitt’s lymphoma Raji, T cell leukemia Jurkat, promyelocytic leukemia HL60, chronic myelogenous leukemia K562, and mouse myeloma Sp2/0-Ag14 were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Burkitt’s lymphoma BJAB was a generous gift from Dr. Fred Wang (Harvard). All of the cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin, streptomycin, and fungizone.

Antibodies—DX2, a mouse mAb (IgG1) specific for an epitope on the extracellular domain of human Fas was obtained from Pharmingen (San Diego, CA). CH11, a mouse mAb (IgM) specific for an epitope on the extracellular domain of human Fas was purchased from PanVera (Madison, WI). Goat anti-mouse IgM used for immunoprecipitation was obtained from Cappel (Durham, NC). Fas/C-20 (rabbit antiseraum recognizing the carboxyl terminus of Fas (amino acids 300–319), Fas/N-18 (rabbit antiseraum recognizing the amino terminus of Fas (amino acids 5–52)), and PARP/N-20 (goat antiseraum recognizing the N terminus of PARP (amino acids 1–20)) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CPP32 and anti-FADD were purchased from Transduction Laboratories (Lexington, KY).

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The abbreviations used are: TNFR, tumor necrosis factor receptor; PARP, poly(ADP ribose) polymerase; GST, glutathione S-transferase; PCR, polymerase chain reaction; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; LLM, N-acetyl-l-leucinal-l-leucinal-methional; LLnL, N-acetyl-l-leucinal-l-leucinal-l-norleucinal; ICE, interleukin-1β-converting enzyme.
V. The membrane was blocked at room temperature for 1 h with TBS-T (14).

Deletion mutants ΔN-1 and ΔN-2 were constructed by limiting dilution using reverse primer wt-t and forward primers ΔN-1 (5'-CGATGATCCCTGGTAGCTAGTATGTTTAG-3') and ΔN-2 (5'-CGATGATCCCTGGTAGCTAGTATGTTTAG-3'). Deletion mutants ΔC-1 and ΔC-2 were constructed with forward primer wt-f and reverse primers ΔC-1 (5'-CGATGATCCCTGGTAGCTAGTATGTTTAG-3') and ΔC-2 (5'-CGATGATCCCTGGTAGCTAGTATGTTTAG-3'). LPR point mutant was constructed by PCR as described previously (3).

pGEX-2TK/WT was used as template, and primers wt-t, wt-r, lpr-f (5'-TTCGAAAGATGCTGAATGGAACCCAAA-3'), and lpr-r (5'-TCTGCTTCATGTTAACCTTTCGAA-3') complementary to lpr were used. The PCR products for Fas mutants ΔN-1, ΔN-2, ΔC-1, ΔC-2, and LPR were digested with BamHI and EcoRI and subcloned into pGEX-2TK to generate plasmids pGEX-2TK/ΔN-1, pGEX-2TK/ΔN-2, pGEX-2TK/ΔC-1, pGEX-2TK/ΔC-2, and pGEX-2TK/LPR.

Expression and purification of GST fusion proteins—Extraction and purification of GST fusion proteins were performed as described (14). Escherichia coli BL21 (Stratagene, La Jolla, CA) transformed with pGEX-2TK recombinants was grown logarithmically (500 ml, A\text{sub}500 = 0.8) and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 2.5 h. Cells were then pelleted and resuspended in 50 ml of ice-cold NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 100 μg/ml egg white lysozyme (Sigma). Bacteria pellet was lysed on ice by mild sonication and centrifuged at 40,000 g for 30 min at 4 °C. Bacterial supernatants were mixed for 1 h at 4 °C with 750 μl of glutathione-Sepharose beads (Pharmacia) that had been previously washed three times and resuspended (final concentration, 1:1 (v/v) in NETN). Coated glutathione-Sepharose beads were then washed three times with NETN. For analysis of bound fusion proteins, beads were incubated in a sample buffer containing 2% SDS at 45 °C for 1 h and loaded onto SDS-PAGE gels.

Production of Monoclonal Antibody (Clone 3D5)—Female BALB/c mice were immunized with the cytoplasmic domain of human Fas (Fas-CD). Splicenic cells were fused with mouse myeloma Sp2/0-Ag14 cells, and hybrids were selected in culture medium containing hypoxanthine, aminopterin, and thymidine. Supernatants of hybrid cultures containing 2% SDS at 45 °C for 1 h and loaded onto SDS-PAGE gels.

Flow Cytometry Analysis—Indirect immunofluorescent staining was performed as described (15). 1 × 10^6 cells were incubated with 1.25 μg/ml of DX2 for 30 min on ice followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG (Caltag Laboratories, San Francisco, CA). After fixation with 1% paraformaldehyde, cells were analyzed with a Coulter Cytometry Profile I (Coulter, Hialeah, FL).

Immunoprecipitation—Immunoprecipitation was performed by using glutathione-Sepharose beads coupled with GST fusion proteins to remove antibodies reacting with the fusion proteins. 1 μl of diluted anti-Fas antibody (1:1500) or 1 ml of the supernatant was incubated overnight with GST, GST-Fas-IC-, or GST-FADD-coated beads. Then the bead suspension was centrifuged, and the supernatant was used for Western blotting as a postimmunoprecipitated antibody.

RESULTS

Detection of Human Fas Antigen Using Anti-cytoplasmic Domain Antibodies—Antibodies directed against the extracellular domain of Fas antigen have been used exclusively in the past to study the Fas molecule and its associated proteins. To gain more insight into the biochemical event following Fas activation, antisera and monoclonal antibodies against the cytoplasmic domain of human Fas were generated (see “Experimental Procedures”). Western blot analysis was performed in a panel of human cell lines that expressed different amounts of Fas antigens on their cell surface. As shown in Fig. 1B, Fas antigen could be detected in BJAB, Raji, and Jurkat, but not in K562 and HL60 by antiserum directed against the cytoplasmic domain of Fas. The amount of Fas antigen detected in the Western blot closely resembled the amount detected with an antiextracellular domain mAb by flow cytometry (Fig. 1A). An mAb against the cytoplasmic domain of Fas (clone 3D5) was also applied in Western blot analysis with identical result (data not shown). As shown in Fig. 1B, there was significant heterogeneity in the molecular size of the Fas antigen in different cell lines. The protein backbone of the human Fas has an estimated molecular mass of 36 kDa (16). BJAB and Raji cells expressed two major bands (40 and 50 kDa) and one minor band (42 kDa), whereas Jurkat cells expressed a doublet of 45 and 46 kDa. The heterogeneity in molecular weight is most likely due to differential glycosylation of the two N-linked glycosylation sites in the extracellular domain of Fas (16).
more, 3D5 does not cross-react against several death domain containing proteins, such as FADD/MORT1, CD40, and TNFR1 (data not shown, and see below).

Formation of High Molecular Weight Fas Aggregates—The anti-cytoplasmic domain antibody (3D5) was used to investigate the fate of Fas antigen after stimulation with anti-Fas mAb (Fig. 3A). Three Fas-expressing cell lines, BJAB, Raji, and Jurkat, were chosen for the time course study. To detect Fas by Western blotting, we used a secondary antibody specific for mouse IgG, but not mouse IgM, to avoid detecting the stimulating antibody, CH11 (mouse IgM). All three cell lines showed similar patterns of change following activation. Cross-linking of Fas on the cell surface by CH11 resulted in the formation of high molecular weight Fas aggregates, which were stable in 2% SDS and 5% β-mercaptoethanol (Fig. 3A). These aggregates

**Fig. 1.** Human Fas expression analyzed by fluorescence-activated cell sorting and Western blotting. A, flow cytometric detection of Fas expression using anti-extracellular domain antibody. BJAB, Raji, Jurkat, K562, and HL60 were stained with anti-Fas (DX2) followed by fluorescein isothiocyanate-labeled anti-mouse IgG (closed histogram). The open histogram indicates background staining with the second-step antibody alone. B, Western blot analysis of Fas expression using anti-cytoplasmic domain antibody. Total cell lysates were analyzed by Western blotting using preimmune serum or mouse antiserum against the cytoplasmic domain of human Fas.

**Fig. 2.** Mapping of the epitope detected by monoclonal antibody 3D5. A, schematic diagram of GST fusion proteins containing the mutated cytoplasmic domain of Fas. The entire cytoplasmic domain (residues 175–319) (WT) of Fas, its deletion mutants (ΔN-1, ΔN-2, ΔC-1, and ΔC-2), and the point mutant (LPR) were shown as fusion proteins linked to GST as described under “Experimental Procedures.” The shaded region indicates the death domain. B, GST-Fas mutants stained with Coomassie Blue. GST fusion proteins (100 ng/lane) were subjected to 12% SDS-PAGE and stained with Coomassie Blue. Molecular mass standards are expressed in kDa. C, Western blotting of GST-Fas mutants with hybridoma supernatant of 3D5. GST fusion proteins of WT and the mutants (1 ng/lane) were subjected to 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and incubated with the supernatant of hybridoma (clone 3D5).
were more than 200 kDa in size and could be detected 10 min after incubation with CH11. The appearance of Fas aggregates peaked at 120 min in most cases, whereas Fas monomers disappeared with time. In Jurkat cells, the monomers became undetectable by 120 min.

Generation of p97—In addition to Fas aggregates and monomers, a 97-kDa band (p97) was also detected (Fig. 3A). p97 was detected at 120 min and increased in intensity by 240 min. To compare the molecular mass of p97 in BJAB, Raji, and Jurkat, Western blot analysis was performed on samples derived from these cell lines following CH11 stimulation for 240 min (Fig. 3B). As shown, the 97-kDa bands derived from these cell lines migrated identically.

Immunoabsorption Studies Using Antiserum and Monoclonal Antibody—To confirm the specificity of each band. Diluted mouse anti-Fas antiserum was incubated with beads coated with GST, GST-Fas-CD, or GST-FADD/MORT1. After centrifugation, the supernatant was used as primary antibody for Western blot analysis. D, co-precipitation of the high molecular weight Fas aggregates with FADD/MORT1. Jurkat cells were cultured with anti-Fas (mouse IgM, CH11) for 0, 10, 30, 60, 120, and 240 min. Total cell lysates were immunoprecipitated with anti-FADD monoclonal antibody (mouse IgG), and the precipitates were analyzed by Western blotting using rabbit antiserum specific for the C terminus of Fas (Fas/C-20). The position of the high molecular weight Fas aggregates is indicated by an asterisk. The positions of the heavy and light chains of IgG are also indicated.

Co-precipitation of the High Molecular Weight Fas Aggregates with FADD—Formation of the high molecular weight Fas aggregates requires ligation of the Fas antigen (Fig. 3A). Kischkel et al. and Muzio et al. have demonstrated that the appearance of the Fas aggregate coincides with the formation of a death-inducing signaling complex (2, 9). However, they did not show that association of FADD/MORT1 with Fas requires Fas aggregation. To provide more evidence for the physiologic relevance of the Fas aggregates, Jurkat cells were activated with CH11 as described for Fig. 3A. At the designated time after activation, Jurkat cell lysates were immunoprecipitated with a mouse anti-FADD monoclonal antibody. The immunoprecipitates were then analyzed by Western blotting with a rabbit antiserum specific for the C terminus of Fas. As shown in Fig. 3D, the high molecular weight Fas aggregates could only be co-precipitated with FADD/MORT1 after Fas ligation. This experiment provides direct evidence that the association of FADD with Fas requires Fas aggregation.

p97 Generation Parallels CPP32 Activation and PARP Cleavage—The time course of p97 generation was compared with other known proteolytic events following Fas activation. For this purpose, CPP32 activation and PARP cleavage were analyzed in the same samples used for Western blotting shown in Fig. 3A. The 28-kDa PARP fragment was observed in BJAB, Raji, and Jurkat cells after cross-linking of Fas with CH11 (Fig. 4A). In BJAB, the 28-kDa band was markedly increased 4 h
after incubation with CH11. In Raji and Jurkat, the 28-kDa band was markedly increased 120 min after activation. CPP32 activation was also examined using the same samples described above. The p20 subunit of CPP32 could be detected 240 min after activation in BJAB, and at 120 min after activation in Raji (Fig. 4). Thus, the time course of p97 generation parallels CPP32 activation and PARP cleavage.

p97 Retains the C-terminal Amino Acids of Fas—A rabbit antiserum against the carboxyl-terminal 20 amino acids of Fas (Fas/C-20) was used to further characterize p97. Monomeric Fas molecules could be detected in Raji and Jurkat cells (Fig. 5A and B). However, Fas/C-20 also reacted with several non-specific bands that overlapped with the true monomeric bands (see Figs. 1B and 3A). However, both p97 and Fas aggregates (>200 kDa) could still be detected (Fig. 5). These results indicate that p97 contains the carboxyl terminus of the Fas molecule.

p97 Cannot Be Immunoprecipitated by Anti-extracellular Domain Antibodies—A collection of commercially available antibodies recognizing the extracellular domain of human Fas were used to characterize p97 in Western blot analysis as described by previous reports (16, 17). However, none of these antibodies could detect Fas-specific bands in total cell lysates. The difficulty with using anti-extracellular domain antibodies in Western blot analysis remains to be investigated.

To circumvent the difficulty in Western blot analysis with the anti-extracellular domain antibody, Fas antigen was immunoprecipitated with anti-extracellular domain antibody (Fas/N-18), which was then detected with anti-Fas cytoplasmic domain antibody (3D5) (Fig. 6A, lanes 4–6). As shown in Fig. 6A, Fas monomers could be clearly seen (lanes 4–6), but p97 cannot be seen (lane 6). The high molecular weight Fas aggregates (>200 kDa) were weakly detected in the immunoprecipitated samples (lanes 5 and 6), and their intensities were much lower than those in total cell lysate (lanes 2 and 3). In the immunoprecipitates, however, a broad band of 110–150 kDa was detected (lanes 5 and 6), suggesting that the high molecular weight Fas aggregates might be degraded to smaller proteins (110–150 kDa) during the procedure of immunoprecipitation. In addition to immunoprecipitation with an antiserum directed against the N-terminal 18 amino acids of Fas (Fas/N-18), Fas molecules were also immunoprecipitated with antimouse IgM linked to Protein A-Sepharose beads after incubation with CH11. In Raji and Jurkat, the 28-kDa band was markedly increased 120 min after activation. CPP32 activation was also examined using the same samples described above. The p20 subunit of CPP32 could be detected 240 min after activation in BJAB, and at 120 min after activation in Raji (Fig. 4). Thus, the time course of p97 generation parallels CPP32 activation and PARP cleavage.

p97 can be detected by rabbit antiserum recognizing the carboxyl terminus of Fas (Fas/C-20). Kinetic analysis of Fas processing after CH11 treatment in Raji and Jurkat cells. Cells were cultured with anti-Fas (mouse IgM, CH11) for 0, 10, 30, 60, 120, and 240 min. Total cell lysates were analyzed by Western blotting using Fas/C-20. Horseradish peroxidase-conjugated anti-rabbit IgG was used as a secondary antibody.
immunoprecipitated by goat anti-mouse IgM was loaded in lanes 4 or 240 min. Total cell lysates were loaded in lane 3. Samples immunoprecipitated (IP) by anti-Fas extracellular domain (Fas/N-18) were loaded on lanes 1 and 2. Samples immunoprecipitated by goat anti-mouse IgM was loaded in lane 3. The filters were analyzed by Western blotting (WB) using the anti-cytoplasmic domain monoclonal antibody, 3D5.

**FIG. 6.** p97 cannot be immunoprecipitated by anti-extracellular domain antibodies. A, Raji cells were activated by CH11 for 0, 60, or 240 min. Total cell lysates were loaded on lanes 1, 2, and 3. Samples immunoprecipitated (IP) by anti-Fas extracellular domain (Fas/N-18) were loaded on lanes 4, 5, and 6. B, Raji cells were activated by CH11 for 0 or 240 min. Total cell lysates were loaded in lanes 1 and 2. Sample immunoprecipitated by goat anti-mouse IgM was loaded in lane 3. The filters were analyzed by Western blotting (WB) using the anti-cytoplasmic domain monoclonal antibody, 3D5.

**DISCUSSION**

After cross-linking of Fas with agonistic antibody, high molecular mass Fas aggregates (>200 kDa) were immunoprecipitated with antibodies against the extracellular domain and detected in Western blotting under reducing conditions with antibodies against the death domain or the carboxyl terminus. These results imply that Fas aggregates are composed of a complex of intact Fas monomers. SDS-stable forms of aggregated Fas have been reported by Kischkel et al. (2). Their Fas aggregate has a molecular mass of ~110 kDa. The difference in size of the Fas aggregates in these studies is most likely due to difference in sample preparation. We used Western blot analysis to detect the aggregate, whereas Kischkel et al. used immunoprecipitation. Thus, the 110-kDa band detected by Kischkel et al. probably resulted from protein degradation during the long incubation period of immunoprecipitation (2). This is consistent with our immunoprecipitation results, which demonstrated the presence of both the large (>200 kDa) and small (~110-kDa) Fas aggregates (Fig. 6, A and B).

Following formation of Fas aggregates (>200 kDa), a novel band, p97, was observed. In previous studies, p97 could not be detected because only anti-extracellular domain antibodies were used. p97 was detected by Western blotting with two different anti-Fas antibodies, 3D5 and Fas/C-20, indicating that p97 contained the cell death domain of Fas and the carboxyl terminus. Based on the kinetics of the appearance of different forms of Fas, p97 most likely originated from the Fas aggregates (>200 kDa). Considering the heterogeneity in molecular weight of Fas monomers in different cell lines, it is surprising that p97 has a constant molecular weight in all cell lines examined so far. The best explanation is that p97 is generated by the removal of extracellular domain of Fas aggregates. By removing the differentially glycosylated extracellular domain, the processed Fas aggregates from different cell lines will have uniform molecular mass. Cleavage of the extracellular domains of Fas is suggested by the observation that p97 cannot be precipitated by anti-N terminus antibodies. On the other hand, Fas monomers and Fas aggregates could be readily precipitated. At this moment, we do not know the exact cleavage site of the Fas aggregate in p97 generation. Soluble Fas molecules have been described previously and have been used as indicators of inflammation (19). Up to five different splice variants of Fas that result in truncated and soluble Fas proteins have been reported (20). p97 is unlikely to be the translation product of Fas splice variants because p97 contains the cytoplasmic domain.

It has been previously reported that calpain and ICE family members were activated during Fas-mediated apoptosis (21, 22). We also had evidence that CPP32 and PARP, a known CPP32 substrate, were cleaved in Jurkat cells after cross-linking of Fas. Based on kinetic study, CPP32 activation, PARP cleavage, and p97 generation occur at a similar time. Therefore, Fas aggregates may be cleaved by activated proteases such as proteasome, calpains, lysosomal cysteine proteases, or ICE family members to generate p97. We have seen the involvement of proteases in the p97 generation, protease inhibitors were used to inhibit p97 generation. LLM and LNNL were used as the inhibitors of calpains, lysosomal cysteine proteases, or proteasome (23, 24). The z-VAD-CH₂F and z-DEVD-CH₂F were also used as the inhibitors of ICE family members (25, 26). These protease inhibitors had no inhibitory effect on the p97 generation, suggesting that p97 is generated by protease(s) with other specificity following Fas receptor activation.

The significance of p97 generation is not known. It could be
a byproduct of the proteolytic milieu generated by the activation of ICE/CED3 family of cysteine proteases during apoptosis induction. However, p97 generation could not be blocked by cysteine protease inhibitors. Alternatively, p97 is generated by a novel protease that is activated upon Fas receptor ligation. Shedding of membrane proteins, such as interleukin-6 receptor, L-selectin, and TNFRs, has been reported (27–29). These cleavages appeared to be mediated by novel endopeptidases. It is currently not known whether p97 is generated by similar proteolytic cleavage. The biological role of p97 also warrants further investigation. The current concept of Fas-induced apoptosis invokes the formation of the death-inducing signaling complex that includes the Fas aggregates, FADD/MORT1, and FLICE/MACH. p97 could participate in apoptosis signaling. Alternatively, p97 generation could down-modulate cell death signals. Further experiments are required to elucidate the significance of p97 generation. In summary, a newly generated mAb recognizing the death domain was used to detect a novel form of processed Fas. This antibody could also be used in future studies to identify the biology and cellular fate of the cytoplasmic domain of Fas during apoptosis signaling.

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FIG. 7. Effect of protease inhibitors on p97 generation. A, Western blotting of Jurkat total cell lysates to detect p97 generation in the presence of protease inhibitors. Cells treated with protease inhibitors were stimulated with CH11 for 4 h. Total cell lysates were analyzed by Western blotting using 3D5 or Fas/C-20. B, Western blotting of Jurkat total cell lysates to detect PARP cleavage under the influence of protease inhibitors. Cells pre-treated with protease inhibitors were stimulated with CH11 for 4 h. Total cell lysates were analyzed by Western blotting using a goat antiserum recognizing the N terminus of PARP. The open arrow indicates PARP, and the closed arrow indicates the 28-kDa cleaved product of PARP.
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