Distinct roles of haptoglobin-related protein and apolipoprotein L-I in trypanolysis by human serum

Benoit Vanhollebeke*, Marianne J. Nielsen†, Yoshihisa Watanabe‡, Philippe Truc§, Luc Vanhamme*, Kazunori Nakajima*, Soren K. Moestrup†, and Etienne Pays*

*Laboratory of Molecular Parasitology, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 12, Rue des Profs Jeener et Brachet, 86041 Gosselies, Belgium; †Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus, Denmark; ‡Japanese Red Cross Central Blood Institute, Tokyo 135-8521, Japan; §Institut de Recherche pour le Développement, Unité de Recherche 117 Trypanosomoses Africaines, Instituto de Combate e Controlo das Tripanosomioses, CP 2657 Luanda, Angola; and ¶Tokyo Red Cross Blood Center, Tokyo 135-8639, Japan

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African trypanosomes, the prototype of which is Trypanosoma brucei (T. b. brucei), are protozoan parasites transmitted by tsetse flies. Living extracellularly in the bloodstream of their mammalian hosts, they overcome host adaptive immunity by restricting their exposed immunogenic epitopes to a continuously changing coat of variant surface glycoprotein (1). Humans and some primates exhibit specific innate immunity that allows them to kill T. b. brucei, but not Trypanosoma brucei gambiensense and Trypanosoma brucei rhodesiensense. These last subspecies are responsible for the sleeping sickness disease, which affects >300,000 people per year in sub-Saharan Africa.

The trypanosome lytic factor (TLF) of normal human serum (NHS) was found to be associated with a minor subclass of high-density lipoproteins (HDL) that contains both haptoglobin-related protein (Hpr) and apolipoprotein L-I (apoL-I) (2–4). Trypanolysis results from the endocytosis of these particles by the parasite (5–7). Initially, the lytic component of TLF was identified as Hpr, and its effect on trypanosomes was attributed to oxidative damage of the lysosomal membrane (8). More recently, apoL-I was shown to exert trypanolytic activity by forming pores into the lysosomal membrane of the parasite, triggering fatal osmotic ionic fluxes (9, 10). The role of apoL-I in trypanolysis could be evidenced with both native and recombinant apoL-I (9–12). In contrast, the involvement of Hpr in this process largely was deduced from indirect evidence resulting from the difficulty of producing recombinant heterodimeric Hpr. Nevertheless, recent work concluded that affinity-purified Hpr was toxic for trypanosomes, leading to the reporting that apoL-I and Hpr are two inefficient toxins whose activities need to be combined to build the trypanolytic potential of NHS (11). However, this view was debated (12).

Apart from a proposed involvement in trypanolysis, the biological function of Hpr and apoL-I is not clear. Hpr shares 91% amino acid sequence identity with Hp, an abundant (0.2–2 mg/ml) acute-phase serum protein that binds free Hb with high affinity and allows its clearance from the blood (13). Recently, the capacity of Hpr to bind Hb was demonstrated (14). However, Hpr probably does not function to scavenge Hb because, in contrast to haptoglobin (Hp), this protein is not cleared from the circulation during intravascular hemolysis (13). In addition, Hpr and Hp strongly differ in their ability to associate with apolipoprotein A-I (apoA-I)-containing HDL particles (14, 15). ApoL-I is the only secreted member of a family that could be involved in programmed cell death (16). It contains a pore-forming domain resembling that of bacterial colicins and Bel-2 family members, as well as a region necessary for the membrane insertion of this pore-forming domain (10, 12).

We describe the trypanolytic potential of mutant human sera lacking either Hpr or apoL-I. Human serum devoid of Hp and Hpr [Hp(r)−/−HS] originates from anhaptoglobinemic patients lacking both Hpr and Hp as a result of homozygous allele. Here, we show that this serum, devoid of any trypanolytic activity, exhibits normal concentrations of HDL-bound Hpr. Conversely, the serum of individuals with normal HDL-bound apoL-I but who lack Hpr and haptoglobin [Hp(Hp(r)−/−HS) as the result of gene deletion (anhaptoglobinemia)] exhibited phenotypically normal but delayed trypanolytic activity. The trypanolytic properties of Hp(r)−/−HS were mimicked by free recombinant apoL-I, whereas recombinant Hpr did not affect trypanosomes. The lysis delay observed with either Hp(r)−/−HS or recombinant apoL-I could entirely be attributed to a defect in the uptake of the lytic components. Thus, apoL-I is responsible for the trypanolytic activity of normal human serum, whereas Hpr allows fast uptake of the carrier HDL particles, presumably through their binding to an Hp/Hpr surface receptor of the parasite.

Apolipoprotein L-I (apoL-I) is a human high-density lipoprotein (HDL) component able to kill Trypanosoma brucei brucei by forming anion-selective pores in the lysosomal membrane of the parasite. Another HDL component, haptoglobin-related protein (Hpr), has been suggested as an additional toxin required for full trypanolytic activity of normal human serum. We recently reported the case of a human lacking apoL-I (apoL-I−/−HS) as the result of frameshift mutations in both apoL-I alleles. Here, we show that this serum, devoid of any trypanolytic activity, exhibits normal concentrations of HDL-bound Hpr. Conversely, the serum of individuals with normal HDL-bound apoL-I but who lack Hpr and haptoglobin [Hp(r)−/−HS] as the result of gene deletion (anhaptoglobinemia) exhibited phenotypically normal but delayed trypanolytic activity. The trypanolytic properties of Hp(r)−/−HS were mimicked by free recombinant apoL-I, whereas recombinant Hpr did not affect trypanosomes. The lysis delay observed with either Hp(r)−/−HS or recombinant apoL-I could entirely be attributed to a defect in the uptake of the lytic components. Thus, apoL-I is responsible for the trypanolytic activity of normal human serum, whereas Hpr allows fast uptake of the carrier HDL particles, presumably through their binding to an Hp/Hpr surface receptor of the parasite.
sequence, was found to be entirely normal (data not shown).
Conversely, Hp(r)/−/− HS was devoid of both Hp and Hpr while
containing normal levels of HDL-bound apo-L-I (Fig. 1).

Trypanosome survival assays were conducted over the course of
24 h in either 10% normal or mutant human sera (Fig. 2A). This
long incubation period (approximately four generation times) was
chosen to allow the detection of even traces of trypanolytic or
trypanostatic activity. As reported previously (20), apo-L-I/−/− HS
was completely devoid of any trypanolytic activity because the
number of trypanosomes living after 24 h of incubation in that
serum was similar to that in fetal calf serum (FCS), which is nonlytic
and contains neither apo-L-I nor Hpr. Even in 100% apo-L-I/−/− HS,
trypanosomes grew as fast as in FCS (data not shown), whereas the
trypanolytic activity of NHS could still be monitored after a 10^5
dilution (Fig. 2B). However, the absence of Hpr had no effect on
trypanosome survival because, as in NHS, the entire trypanosome
population was lysed in Hp(r)/−/− HS (Fig. 2A). In both FCS and
apo-L-I/−/− HS, trypanolytic activity was restored after the addition
of physiological levels (8.5 µg/ml) of recombinant apo-L-I. In
contrast, no lytic activity was demonstrated in FCS after the
addition of even a 5-fold excess of recombinant Hpr that exhibited
the natural processing characteristics of this protein into α-chains and
β-chains as well as full capacity of Hb binding (14), although it
was devoid of the hydrophobic signal peptide present in plasma-
derived Hpr (Fig. 2A).

Hp(r)/−/− HS Exhibits Normal But Delayed Trypanolysis. The pheno-
type of trypanolysis by NHS or Hp(r)/−/− HS was indistinguish-
able. In both cases, only NHS-sensitive, and not NHS-resistant,
trypanosomes were lysed (Fig. 2B). Moreover, in each case, lysis
typically was preceded by considerable lysosomal swelling (Fig.
3A). This effect is known to depend on active endocytosis,
adeciduation of endosomes (5–7), and massive influx of chloride
ions (10). Accordingly, in both sera, trypanolysis could be
blocked at 4°C, inhibited by lysosomotropic basifying agents such as
chloroquine, and prevented by the anionic channel inhibitor
4,4-diisothiocyanatostilbene-2,2-disulfonic acid (Fig. 3B).

Despite these phenotypic similarities, the absence of Hpr led to a
significant delay in the trypanolytic process, particularly at
high serum dilution. When incubated in 100% serum, the ratio
in population midlysis time between Hp(r)/−/− HS and NHS was
≈1.65 (±0.11; n = 3), and this ratio reached 2.21 (±0.14; n =
3) in 30% serum (Fig. 3C). This effect was assessed by incubating
trypanosomes in logarithmic dilutions of both sera. Although
after 24 h of incubation lytic activity could still be detected after
a 10^5 dilution of NHS, lysis did not occur at >10^3 dilution of
Hp(r)/−/− HS (Fig. 3D). This trypanolytic potential was close to
that obtained with equivalent levels of recombinant wild-type
apo-L-I directly added to FCS (Fig. 3D).

Hpr Mediates the Binding of TLF to a Speciﬁc Surface Receptor. Taking advantage of the irreversibility of the lysis process (10),
we preincubated trypanosomes at 37°C for increasing periods of
time in 10% different human sera. Then, after washing, we
resuspended them in 10% nonlytic FCS and incubated them for
24 h at 37°C. These experiments indicated that the parasite
commitment to death (the minimal preincubation time required
to trigger lysis) was reached 25 times quicker in NHS than in
Hp(r)/−/− HS [midvalues of 5.0 min (±0.5; n = 3) versus 126.1 min
(±19.9; n = 3) in 10% serum; Fig. 4A]. The same experiments

![Figure 1](image1.png)

**Fig. 1.** Characteristics of apo-L-I−/− and Hp(r)−/− human sera. NHS, apo-L-I−/− HS, and Hp(r)−/− HS were fractionated by immunoaffinity chromatography on an anti-apo-L-I column in the absence of detergents. (A) Coomassie blue staining of equivalent volumes of unfraccionated serum (W, whole), apo-L-I depleted serum (FT, flow-through), and bound fraction (BF). The identity of apoA-I was checked by immunodetection (data not shown). (B) Immunodetection of apo-L-I, Hp α2 chain, and Hpr α chain.

![Figure 2](image2.png)

**Fig. 2.** Trypanolytic potential of various sera. The trypanosomes, isolated from mice, were incubated at 1.10^10/ml in HMI-9 for 24 h with 10% of the indicated serum. After 24 h, living trypanosomes were counted in triplicate under the microscope. In this and Figs. 3–6, errors bars represent SD from three independent experiments, and the trypanosome concentrations are normalized to growth in 10% NHS. Growth of ETat 1.2R in 100% NHS was normalized to that in 100% FCS. (A) ETat 1.2S (NHS-sensitive) trypanosomes were incubated in various sera containing or not containing 8.5 µg/ml free recombinant apo-L-I or 150 µg/ml free recombinant Hpr. (B) ETat 1.2S and ETat 1.2R (NHS-resistant) trypanosomes were incubated in various serum concentrations. At serum concentrations <10%, FCS was added to maintain a final 10% serum concentration.

![Figure 3](image3.png)

**Fig. 3.** Characteristics of apo-L-I−/− and Hp(r)−/− human sera. NHS, apo-L-I−/− HS, and Hp(r)−/− HS were fractionated by immunoaffinity chromatography on an anti-apo-L-I column in the absence of detergents. (A) Coomassie blue staining of equivalent volumes of unfraccionated serum (W, whole), apo-L-I depleted serum (FT, flow-through), and bound fraction (BF). The identity of apoA-I was checked by immunodetection (data not shown). (B) Immunodetection of apo-L-I, Hp α2 chain, and Hpr α chain.
were conducted after preincubating the trypanosomes at 4°C, a temperature low enough to completely inhibit lysis and endocytosis (5). In the case of NHS, no significant difference in trypanolysis could be demonstrated after preincubation at either 37°C or 4°C. In contrast, in the case of Hp(r)−/−HS, trypanolysis was no longer detectable after preincubation at 4°C (Fig. 4A). Similar results were obtained by using FCS supplemented with free recombinant apoL-I (Fig. 4A). These data revealed that the absence of Hpr prevents the binding of TLF to trypanosomes at 4°C, suggesting that Hpr is required for the binding of TLF to a specific surface receptor.

To assess this hypothesis, we tested the effect on trypanolysis of adding a constant amount of free Hp or Hpr (200 μg/ml) to various dilutions of NHS. As expected from previous reports analyzing the effect of Hp on the activity of purified lytic particles (21–23), an excess of Hp was able to inhibit some, but not all, trypanolytic activity of NHS (Fig. 4B). Very similar results were obtained with recombinant Hpr (Fig. 4B). Therefore, Hp and Hpr appeared to interfere with TLF activity only when present in large excess, as would occur if these proteins were competing with TLF for binding to the trypanosome surface. That this effect was specific was indicated by the lack of inhibition observed with both proteins added to Hp(r)−/−HS (Fig. 4B). Thus, these results further suggested the involvement of Hpr in the binding of HDL to the trypanosome surface. Such a conclusion was independently reached previously (24) and also was supported by the monitoring of intracellular trafficking of free Hp (Fig. 5). Hp appeared to rapidly and efficiently accumulate in the endocytic pathway, contrasting with the inefficient uptake of bovine serum albumin (BSA), a protein not specifically recognized by the parasite (Fig. 5).

The Mechanism of Lysis Does Not Involve Hpr. To assess the hypothesis that apoL-I and Hpr act synergistically to generate full trypanolytic activity (11), we plotted the relationship between the time periods necessary in various NHS concentrations to achieve commitment to lysis (as determined by experiments involving preincubation with the lytic sera then incubation in nonlytic FCS; see Fig. 4A) and those necessary to observe complete lysis. The data, shown in Fig. 6, indicate that the time of lysis by NHS is related to the time required for commitment to lysis, both depending on the concentration of serum. This effect could be ascribed to later and longer times of lysosomal swelling when the number of internalized apoL-I molecules is reduced. This relationship also was measured for trypanolysis triggered by Hp(r)−/−HS. As shown in Fig. 6, irrespective of the period required for cellular commitment to lysis, the time necessary for lysis after the commitment period was never longer in the absence of Hpr than in NHS. Again, similar results were obtained with FCS + recombinant apoL-I (Fig. 6). Thus, regardless of the presence or absence of Hpr, the lysis time could be predicted by that required for commitment to lysis, and the longer time of trypanolysis observed with Hp(r)−/−HS could totally be assigned to slower uptake of the lytic component and not to the decrease in lytic activity. These data contradict the idea that apoL-I and Hpr work in synergy.

Discussion

The identification of the trypanolytic factor of NHS has been highly controversial. Two different HDL-bound serum proteins specific to humans, Hpr and apoL-I, successively have been proposed as trypanosome toxins, and the mechanisms by which these factors were thought to kill trypanosomes have varied from membrane lipid peroxidation to ionic pore-forming activity [for
a recent review, see Pays et al. (12)]. Currently, the trypanolytic potential of apoL-I is recognized generally, but the role played by Hpr is still debated. It was recently proposed that Hpr and apoL-I are both weakly cytotoxic to *T. brucei*, but that their specific activities for killing increase several hundredfold when assembled in the same HDL particles (11).

We addressed this issue by analyzing the trypanolytic potential of human sera naturally devoid of either apoL-I or Hpr. Moreover, we evaluated the trypanolytic capacity of pure recombinant Hpr, apparently correctly processed and functional with regard to Hb binding, although lacking the hydrophobic signal peptide (14). The data failed to demonstrate any trypanolytic activity of Hpr alone, whether recombinant or naturally present in HDL particles. These results confirmed those obtained in transgenic mice expressing Hpr because, despite the presence of HDL-bound Hpr, these mice appeared to be normally infected by *T. b. brucei* (25). In contrast, the absence of apoL-I alone was necessary and sufficient to completely eliminate the trypanolytic potential of NHS, whereas the addition of recombinant apoL-I alone was necessary and sufficient to confer this potential to nonlytic sera. The phenotype of trypanosome lysis by apoL-I alone, whether in its natural HDL context or added as recombinant protein, was indistinguishable from that by NHS. However, in both cases, a clear and similar delay of trypanolysis was observed. Despite this delay, Hp(r)/H11002/H11002/H11002 HS was still fully trypanolytic after 10/11003 dilution, indicating that the absence of Hpr should not affect the natural host resistance to *T. b. brucei* infection. These results indicated that apoL-I is the trypanolytic factor, but they also confirmed previous reports concluding that Hpr is involved in trypanolysis (8, 11, 21–23).

More precisely, these data showed that Hpr allows TLF to act faster. To dissect this effect, we conducted trypanolysis experiments dissociating TLF uptake and lysis by preincubation for different periods and different temperatures in lytic sera, followed by cell washing and incubation in nonlytic FCS. These experiments revealed that the absence of Hpr, whether in Hp(r)/H11002/HS or FCS supplemented with recombinant apoL-I, was linked to the incapacity of TLF to penetrate trypanosomes at 4°C. The simplest interpretation of these data is that the presence of Hpr in the trypanolytic particles allows them to bind to trypanosomes at 4°C, a characteristic of ligands interacting with a specific surface receptor. This interpretation was confirmed by specific competition by excess of free recombinant Hpr or free Hp, in accordance with previous reports showing that Hp inhibits the trypanolytic activity of purified TLF particles (21–23). Therefore, it is highly probable that Hpr allows TLF to bind to a specific Hp/Hpr receptor of the parasite surface, as already
proposed (24). A scavenger receptor for lipoproteins also has been suggested to be involved in TLF uptake (26). Because TLF binding to trypanosomes appeared to involve two receptors, one present in 350 copies exhibiting high affinity and another present in 60,000 copies binding with low affinity (24), a combination of specific and scavenger receptors were required for optimal uptake of TLF. Only incomplete inhibition of trypanolysis was observed with large excess amounts of Hp or Hpr, but this finding cannot be simply explained by the existence of two different receptors. Indeed, a significant fraction of the lytic component that resisted excess Hp(r) actually depended on Hpr, as revealed with Hp(r)-free serum. The characteristics of this fraction evoke those of the lytic subfraction TLF2 (22, 23). How Hpr appears to be inaccessible to competing free Hp(r) within this subfraction remains a mystery.

The presence of an Hp receptor on the trypanosome surface would be useful for heme and iron uptake by the parasite because it would allow internalization of bound Hb. That this interpretation is plausible is indicated by results obtained when monitoring the growth of trypanosomes in transferrin-depleted medium. Indeed, trypanosomes thought to lack efficient TLF uptake, such as NHS-resistant clones of T. b. rhodesiense (27), do not tolerate deprivation of transferrin, in contrast to T. b. brucei parasites, which survive despite a strong reduction of growth (28). A possible explanation of this difference is that uptake of iron through the Hp receptor would compensate for the inability to capture iron from transferrin. The putative Hp receptor is expected to recognize Hpr-containing HDL particles, which also contain Hpr-bound Hb (14). Thus, the uptake of Hpr–Hb-containing HDL particles could contribute to providing a source of heme to the parasite, which lacks the pathway for heme biosynthesis (29). According to these views, internalization of apoL-I by the parasite would be a byproduct of heme uptake from Hpr–Hb-containing HDL particles.

The time necessary for complete lysis of trypanosomes by NHS could essentially be predicted by the time required to irreversibly commit them to lysis. As indicated by the lack of difference between 37°C and 4°C in the case of NHS, the period necessary to commit trypanosomes to death is likely to represent that required for binding and uptake of the lytic factor, the rest accounting for the lytic process itself. This interpretation is supported by the observation that the swelling of the lysosome always becomes detectable soon after the end of the period of commitment to death, irrespective of the length of this period (B.V., data not shown). The TLF uptake time increased with dilution of serum and was thus dependent on the relative abundance of TLF. Whereas TLF was taken up at a speed (2 min in 30% NHS) comparable with that of transferrin (30), the important increase of uptake time observed after dilution of serum (5 min in 10% NHS) suggests that TLF is not in excess in NHS and/or that the trafficking of TLF to the lysosome membrane is relatively inefficient. Presumably, many apoL-I molecules are degraded before reaching their target.

Regarding the lytic process, because apoL-I generates ionic pores that allow an influx of chloride ions into the lysosome (10)), it is logical to assume that the greater the number of intracellular apoL-I molecules, the sooner and the faster lysosome swelling and cell lysis will be. It is likely that at high serum concentration the lytic process is initiated before the intracellular trafficking of apoL-I is complete because in >5% serum concentration the time for commitment to lysis was sharply shortened with respect to total lysis time. This view is actually supported by two observations: Preincubation of trypanosomes with NHS under conditions preventing full intracellular trafficking (that is, at 17°C) still allowed initiation of lysis (B.V., data not shown); moreover, the T. b. rhodesiense SRA protein was found able to block TLF activity upstream from the lysosomal compartment (31).

Taking these different parameters into consideration, we detailed the time period required to lyse trypanosomes with Hpr-free sera, either Hp(r) 175 HS or FCS + apoL-I. The time of commitment to lysis by these sera (presumably the time required for TLF uptake) was clearly longer than in NHS, in full agreement with a putative absence of ligand normally involved in efficient binding of TLF to a specific surface receptor. However, in these sera the process of lysis was never slower than normal, as determined by plotting the lysis times against the times required for TLF uptake with different concentrations of serum. Thus, the delay of trypanolysis linked to the absence of Hpr could entirely be attributed to inefficiency of TLF uptake, contradicting the possibility that Hpr is required to synergize the lytic activity of apoL-I (11). In conclusion, within the trypanolytic HDL particles Hpr and apoL-I appear to be respectively involved in efficient uptake and lysis, and apoL-I is probably the sole trypanolytic factor of human blood.

Materials and Methods

Trypanolysis Assays. A NHS-sensitive clone of T. b. rhodesiense (ETat 1.2S) was used in all experiments. Trypanosomes, isolated from mice, were incubated at densities between 1.10^6 and 1.10^7/ml in HMI-9 medium (32) at 37°C in a CO2-equilibrated incubator. At the indicated times, living trypanosomes were counted in triplicate under the microscope (three independent experiments). The specificity of the lytic activity was systematically checked by verifying the absence of lysis in another clone expressing the same variant surface glycoprotein (ETat 1.2 R) but resistant to NHS because of the synthesis of SRA (33).

Kinetics of TLF Uptake. Trypanosomes, isolated from mice, were incubated at 1.10^6/ml in HMI-9 or PSGS [2.5 mM NaH2PO4。H2O/47.5 mM NaCl/0.365 mM NaClI/1.5% wt/vol glucose/4.4% wt/vol sucrose, pH 8] under the indicated experimental conditions. Aliquots (100 μl) were centrifuged at 7,200 × g for 2 min at either 37°C or 4°C. Pelleted cells carefully were washed twice with HMI-9 medium supplemented with 10% FCS, resuspended in 1 ml HMI-9 + 10% FCS, and incubated for 24 h at 37°C in a CO2-equilibrated incubator before being counted in triplicate under the microscope.

ApoA-I Immunoaffinity Chromatography and Western Blotting. A total of 400 μg of rabbit anti-human apoA-I IgGs (Calbiochem, San Diego, CA) was incubated with 200 μl of washed Affi-Gel 10 (Bio-Rad, Hercules, CA) for 90 min at 4°C. Remaining active esters were blocked with 1 ml of 100 mM ethanolamine-HCl (pH 8) before extensive PBS washes. A total of 60 μl of gel was mixed with 400 μl of 20 × diluted NHS in PBS for 180 min at 4°C. Aliquots of unfraccionated serum, flow-through, and bound fraction were subjected to SDS/PAGE. Western blots were incubated overnight at 4°C with a 1:100 dilution of a goat polyclonal monospecific anti-apoL-I antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or with a 1:10,000 dilution of rabbit polyclonal anti-human Hpr antibody (DAKO, Carpinteria, CA) in 150 mM NaCl/0.5% wt/vol Tween 20/20 mM Tris-HCl (pH 7.5) with 1% nonfat milk. The secondary antibodies were, respectively, peroxidase-conjugated mouse anti-goat IgGs (1:160,000; Sigma–Aldrich, St. Louis, MO) and peroxidase-conjugated mouse anti-rabbit IgGs (1:5,000; Promega, Madison, WI).

DNA Amplification and Sequencing. Genomic DNA was extracted from peripheral blood cells. Five different primer sets (from 5’ to 3’): CAGGTCCAAAGTTTTGACACAGG and TTTCGTACATGCACCAAAATGATGC; GCATGGTCGTG-GAAGCAGGGAGACC and CATCATGGAATGCTG CAGAGGG; TGGCTTCTCACTGTGGTTCG; and GAAAGCAGTGCTCTTGGAGCTTCC; TCACCCATTTCTCAGATGGAAAGGC and TGGCAATCGATTGTT-
CAGCCACAGG; and AGAGAGTGATGCCCATCTGC-CTACC and TATCGCATCCTCCTGCTCCATCC) were used to amplify the Hpr coding sequences from 100 ng of genomic DNA with Pwo DNA polymerase (Roche Diagnostics, Basel, Switzerland). Sequencing was conducted directly on the amplification products.

**Preparation of Recombinant Proteins.** Recombinant apoL-I and Hpr were prepared as described, respectively, in Vanhollebeke et al. (20) and Nielsen et al. (14). Their purity was checked by SDS/PAGE.

**Immunofluorescence.** After incubation in 30% NHS, Hpr(r)−/HS, and FCS for 150, 360, and 360 min, respectively, PBS-washed cells were fixed in 3.7% paraformaldehyde for 10 min at 20°C before being spread on poly(l-lysine)-coated slides and subsequently treated with 0.1% (vol/vol) Triton X-100 in Tris-buffered saline for 5 min at 20°C. p67 was detected with a 1:1,000 dilution of monoclonal anti-p67 antibody (mAb280; J. Bangs, D. Russell). Primary antibodies were detected with an Alexa (488)-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR). Cells were examined with a Zeiss Axiosplan 2 epifluorescence microscope equipped with a Zeiss AxiosCam HRm digital camera (Carl Zeiss, Thornwood, NY).

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