acrylamide gels (75:1 acrylamide:bisacrylamide) in 50 mM tris (pH 8.0) and 400 mM glycine buffer at 250 V for 1 hour. The proteins were expressed and purified as described (*13*). The protein concentrations were determined by absorbance at 280 nm with the use of extinction coefficients of 11,200 M^{-1} cm^{-1} for Sp1 and 2840 M^{-1} cm^{-1} for ZF-QQR. For each protein–nucleic acid pair, a pilot experiment was performed to estimate the dissociation constant. Then, five different concentrations of the protein, centered around the dissociation constant, were used to determine binding affinity. Four independent gel shift experiments were carried out for each protein–nucleic acid pair. The binding affinities determined from the independent experiments were found to be within 20% of one another. The radioactive signal was quantitated with PhosphorImager (Molecular Dynamics) analysis, and the data were fit with the use of the program KaleidaGraph (Synergy Software, Reading, PA).

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15. The purine-rich strand is thought to be the more contacted strand because of the presence of five guanines, the first three of which, in analogy to the Zif268-DNA structure, are presumed to interact with the third zinc finger domain of ZF-QQR through arginine and histidine residues. This inference is also supported by the methylation interference studies, which indicated that methylation of any of these five guanines abolished DNA binding (Y. Shi and J. M. Berg, unpublished data).
16. Y. Shi and J. M. Berg, unpublished data.
17. A single-stranded DNA, 5′-CTCATGTCACTGGGGAAGAAGAAGAATCGATCTGATC-3′, was labeled at its 5′ end with [^{32}P]ATP, hybridized to its complementary RNA strand, and purified from 10% native acrylamide gel as described (*10*). The methylation interference assay was carried out as described [(*22*), pp. 12.3.1–12.3.4].
18. A single-stranded RNA, 5′-GAUCAGAUCGAUUCUUCUUCUUCCCCAGUGACAUGAG-3′, was labeled at its 5′ end with [^{32}P]ATP, hybridized to its complementary DNA strand, and purified as described (*10*). The RNase H protection assay was performed at 25°C for 2 min in a 100-μl reaction buffer containing 40 mM tris (pH 7.5), 100 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, BSA (30 μg/ml), and poly(dI-dC) (2 μg/ml). One unit of *E. coli* RNase H (Pharmacia) was used for each reaction. The reaction was quenched with 400 μl of RNase H stop solution containing 20 mM EDTA, 92% ethanol (v/v), 750 mM ammonium acetate, and 4 μg of poly(dI-dC) at −70°C for 20 min. The precipitated probe was run on a 10% denaturing acrylamide gel.
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Killing of Trypanosomes by the Human Haptoglobin-Related Protein

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African trypanosomes cause disease in humans and animals. *Trypanosoma brucei brucei* affects cattle but not humans because of its sensitivity to a subclass of human high density lipoproteins (HDLs) called trypanosome lytic factor (TLF). TLF contains two apolipoproteins that are sufficient to cause lysis of *T. b. brucei* in vitro. These proteins were identified as the human haptoglobin-related protein and paraoxonase–arylesterase. An antibody to haptoglobin inhibited TLF activity. TLF was shown to exhibit peroxidase activity and to be inhibited by catalase. These results suggest that TLF kills trypanosomes by oxidative damage initiated by its peroxidase activity.

Trypanosomes are protozoan parasites of medical and veterinary importance. In humans, *T. brucei gambiense* and *T. brucei rhodesiense* cause African sleeping sickness. In animals, the disease *nagana* is caused by the parasite *T. b. brucei*. These three subspecies of trypanosomes are morphologically indistinguishable and differ only in their host range and pathologic presentation. The difference in host specificity is caused by a nonimmune killing factor in human serum; the African sleeping sickness trypanosomes are resistant to this factor (*1*).

The trypanocidal activity of normal human serum has been localized to a minor subset of HDLs called TLF, with the majority of serum HDL being nontoxic (*2*). TLF differs from nonlytic HDLs in that it has a higher density (1.21 to 1.24 g/ml) and contains two characteristic apolipoproteins, LI (94.5 kD) and LIII (45 kD) (*2*). LI is composed of three subunits that are 45, 36, and 13.5 kD in size, whereas LIII consists of two apolipoproteins that comigrate, one a 45-kD protein and the other composed of 36- and 13.5-kD subunits. Reconstitution studies have shown that LIII is required for lysis in micellar form in combination with either LI or AI, a major apolipoprotein found in HDLs (*3*).

To establish the identity of LI and LIII, we determined their NH_2-terminal sequences. The subunits of TLF were isolated by two-dimensional gel electrophoresis (*4*) and the individual bands were analyzed by Edman degradation. The LI and LIII 45-kD subunits yielded sequences that were identical to the uncleaved leader sequence of human paraoxonase–arylesterase. The NH_2-termini of the LI and LIII 36-kD subunits were identical to the β subunit of both haptoglobin and the haptoglobin-related protein. The LI and LIII 13.5-kD subunits yielded sequences that were identical (allowing for one sequencing ambiguity) to the uncleaved leader sequence of the α subunit of the haptoglobin-related protein (Fig. 1). Haptoglobin has a similar signal sequence that is normally cleaved at maturation. Haptoglobin is initially translated as a precursor containing the α and β subunits; cleavage at Arg^{84} occurs after a disulfide bond has formed between the two subunits (*5*). By analogy, it is likely that the 36-kD protein is the β subunit of the haptoglobin-related protein.

Human serum paraoxonase–arylesterase associates with HDL_2 and HDL_3 (*6*) and is known to hydrolyze aromatic esters, although its physiological role is unknown. It was an unlikely candidate for the TLF toxin because it is found in HDL_2, which has been shown to be nontoxic (*2*). Haptoglobins are serum glycoproteins that bind free hemoglobin and decrease the loss of iron from the body; however, overloading of the cell with haptoglobin-hemoglobin complexes can cause iron toxicity with the formation of reactive free radicals (*7*). Although it is expressed in amounts that are one-thousandth those of haptoglobin (*8*), the haptoglobin-related protein is predicted to have functional domains that are similar to those of haptoglobin (*9*). Because the haptoglobin-related protein appeared to be a potential toxin, we focused our studies on this protein.

To investigate whether the haptoglobin-related protein was a component of TLF, we measured TLF activity (*2*) in the presence of rabbit polyclonal antibodies to human haptoglobin that cross-react with the haptoglobin-related protein (Fig. 2A). These antibodies inhibited TLF-mediated lysis in a concentration-dependent fashion; this observation implies that the haptoglobin-related protein is part of the lytic molecule. In contrast, nonspecific rabbit polyclonal antibodies had no effect (*10*).

To determine whether the haptoglobin-related protein retained the ability to bind hemoglobin, we assayed for hemoglobin in purified TLF by means of size exclusion chromatography. Total protein absorbance at 280 nm (A_{280}), heme protein absorbance at 407 nm (A_{407}), and TLF lysis activity all coeluted with an apparent molecular mass of 500 kD (Fig. 2B). Hemoglobin was also detected in TLF fractions by immunoblot anal-

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ysis with antisera to human hemoglobin (*10*).

Haptoglobin has peroxidase activity when associated with hemoglobin (*11*). The possibility that TLF also has peroxidase activity was intriguing because the bloodstream forms of the African trypanosomes do not express catalase (*12*); this results in high intracellular concentrations of hydrogen peroxide (H_2O_2). These high concentrations of H_2O_2 make the cells extremely susceptible to oxidative damage (*13*, *14*). Using the procedure of Connell and Smithies (*11*), we detected peroxidase activity in TLF, but not in the nonlytic fraction of human HDLs (Fig. 3A). Peroxidase activity in a sample was proportional to lytic activity (*10*).

We next investigated the possible role of peroxidase activity in TLF-mediated lysis. *Trypanosoma brucei brucei* was incubated in the presence of TLF with increasing concentrations of catalase. Because H_2O_2 freely diffuses through the cell membrane, catalase in the incubation medium should reduce both extracellular and intracellular H_2O_2 concentrations and thus protect the trypanosomes from peroxidase damage. We found that high concentrations of catalase completely inhibited TLF lysis (Fig. 3B), whereas catalase inactivated with 3-aminotriazole and H_2O_2 had no protective effect. Treatment with 3-aminotriazole alone had no effect on either TLF-mediated lysis or trypanosome viability (*10*). These results demonstrate that H_2O_2 is necessary for TLF-mediated lysis, and they suggest that TLF kills the trypanosome by oxidative damage.

We have established the role of the haptoglobin-related protein in the lysis of *T. b. brucei*. The identification of the haptoglobin-related protein in TLF is interesting in itself because it is direct evidence that these proteins are expressed (*8*, *15*). It is also interesting that the haptoglobin-related protein and paraoxonase both contain an uncleaved hydrophobic leader sequence. This finding may represent an important signal in the assembly of proteins into the TLF complex.

TLF and haptoglobin display similar characteristics. TLF is endocytosed and targeted to the lysosome, where it requires an acid pH to lyse trypanosomes (*16*). The haptoglobin-hemoglobin complex is targeted to the lysosomes of liver cells (*17*, *18*) and has maximal peroxidase activity at pH 4 or lower (*11*). The involvement of the haptoglobin-related protein in TLF-mediated lysis is consistent with the observation that only some apes and Old World monkeys have lytic activity in their serum (*19*). The haptoglobin-related protein is the product of a gene triplication event that

45-kD subunit	3 KLIALTLLGMGLALFRNHQ 21	
Paraoxonase–arylesterase	3 KLIALTLLGMGLALFRNHQ 21	
36-kD subunit	1 ILGGHLDAKGSFPWQAKMV 19	
Haptoglobin-related protein (β subunit)	1 ILGGHLDAKGSFPWQAKMV 19	
13.5-kD subunit	3 DLGAVISLLLXGRQLFALY 21	
Haptoglobin-related protein (α subunit)	3 DLGAVISLLLWGRQLFALY 21	

Fig. 1. Sequences of the NH_2-termini of the 45-, 36-, and 13.5-kD subunits of the LI and LIII proteins aligned with human paraoxonase and with the α and β subunits of the human haptoglobin-related protein (*21*). The "X" at position 11 of the 13.5-kD sequence indicates an unreadable amino acid. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

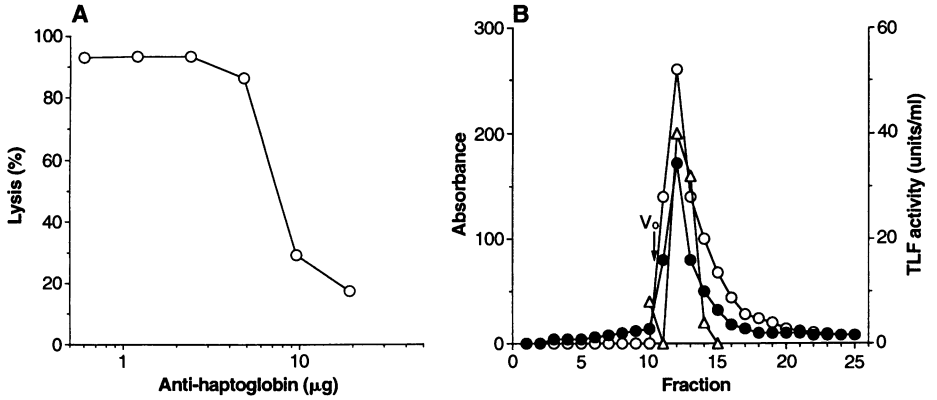

Fig. 2. (**A**) Inhibition of TLF-mediated lysis by antibody to haptoglobin. Rabbit polyclonal antibodies to human haptoglobin (Sigma) were used. The immunoglobulin G fraction of antiserum was isolated and the specificity was established by a single arc of precipitation versus normal human serum and purified human haptoglobin. The lytic assay contained 200 μl of phosphate-buffered saline, 17 units of TLF (2.2 μg), and 100 μl of *T. b. brucei* (3×10^7 cells per milliliter) in F12 media (Gibco) supplemented with 15% fetal bovine serum. The mixture was incubated for 2 hours at 37°C with various amounts of antibodies to haptoglobin. A unit of activity is defined as the amount of TLF necessary to cause 50% lysis in a standard lysis assay. (**B**) Cofractionation of proteins, heme-containing proteins, TLF activity, and peroxidase activity. Purified TLF in phosphate-buffered saline was run over a TSK G3000 SW size exclusion column, and absorbance was measured at A_{280} (proteins; ○) and A_{407} (heme-containing proteins; ●). The silica-based TSK G3000 SW column has a particle size of 10 μm and separates proteins 10 to 500 kD in size. TLF activity (△) from each of the protein-containing fractions was determined as described (*2*). Void (V_o), apoferritin (443 kD), albumin (68 kD), and carbonic anhydrase (28 kD) were used as size standards and eluted in fractions 11, 13, 17, and 23, respectively.

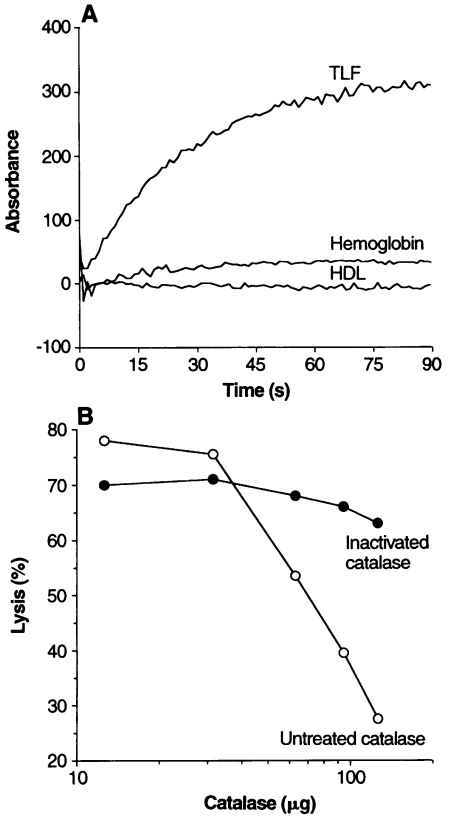

Fig. 3. (**A**) Peroxidase activity of TLF and nonlytic HDLs (*2*). Peroxidase activity was determined (*11*) at pH 4 and 30°C, with 27.5 mM guaiacol as the substrate, and was calculated from the increased absorbance at A_{470} (which signified tetraguaiacol production). The samples contained TLF (300 μg), hemoglobin (2.5 μg), or HDL (300 μg), and H_2O_2 (150 mM) was used to initiate the reaction. (The A_{407}:A_{280} ratio indicated that there was 120 times as much heme in hemoglobin as in TLF.) (**B**) Inhibition of TLF-mediated lysis by catalase. The sensitivity of *T. b. brucei* to TLF was assayed with increasing amounts of untreated catalase (22 U/μg) (○) and with catalase that had been inactivated by treatment with 150 mM 3-aminotriazole and H_2O_2 (●). The catalase concentration used was comparable to that used in other cell systems (*22*). Catalase was inactivated at 26°C by stirring TLF and 3-aminotriazole in a beaker placed in a covered H_2O_2 bath for 24 hours. Untreated catalase was also stirred at 26°C for 24 hours. In this assay, *T. b. brucei* was preincubated with catalase for 1 hour at 37°C before the standard lysis assay (*2*).

occurred in apes and Old World monkeys early in primate evolution, resulting in the haptoglobin gene, the haptoglobin-related gene, and the primate haptoglobin gene (*20*). In humans, a subsequent homologous unequal crossover took place, leaving the original haptoglobin gene and producing the human haptoglobin-related gene, a hybrid of the haptoglobin-related gene and the primate haptoglobin gene. In nonhuman primates, the sequence of the haptoglobin-related protein is known only for chimpanzees, where a frameshift leads to premature termination of translation (*20*). The absence of intact haptoglobin-related protein in chimpanzees is consistent with their lack of TLF activity and may explain why the primate most related to humans does not have this protective mechanism (*19*).

We hypothesize that the haptoglobin-related protein in TLF binds hemoglobin and is endocytosed by the trypanosome, where it is targeted to the lysosome. The low pH of the lysosome stimulates peroxidase activity of the complex, which then reacts with H_2O_2 to cause lipid peroxidation of the lysosomal membrane. The disrupted lysosome then releases its enzymes, and the trypanosome is autodigested.

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Identification of a Dual Specificity Kinase That Activates the Jun Kinases and p38-Mpk2

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One Ras-dependent protein kinase cascade leading from growth factor receptors to the ERK (extracellular signal–regulated kinases) subgroup of mitogen-activated protein kinases (MAPKs) is dependent on the protein kinase Raf-1, which activates the MEK (MAPK or ERK kinase) dual specificity kinases. A second protein kinase cascade leading to activation of the Jun kinases (JNKs) is dependent on MEKK (MEK kinase). A dual-specificity kinase that activates JNK, named JNKK, was identified that functions between MEKK and JNK. JNKK activated the JNKs but did not activate the ERKs and was unresponsive to Raf-1 in transfected HeLa cells. JNKK also activated another MAPK, p38 (Mpk2; the mammalian homolog of HOG1 from yeast), whose activity is regulated similarly to that of the JNKs.

Three distinct protein kinase cascades activate members of the MAPK group in yeast (*1*). One such cascade exists in vertebrates in which Raf activates the MEK group of MAPK kinases (MAPKKs) (*2*), which in turn activate the ERK group of MAPKs (*3*). Raf, however, shares little similarity with the yeast MAPKK kinases (MAPKKKs) STE11 and BCK1, whose mammalian homolog is MEKK (*4*). Several mammalian MAPKs, the JNKs (*5*) or stress-activated protein kinases (*6*) and p38 or Mpk2 (*7*), that are similarly activated by a wide range of physiological and stressful stimuli (*5–9*), have been molecularly cloned. Although MEKK can activate MEK (*4*, *10*), it is a more efficient activator of the JNK cascade, mediating responses to oncogenic Ras and growth factors (*11*). We have now identified a JNK-activating protein kinase, JNKK, that acts between MEKK and JNK. JNKK also activates p38, thus explaining the similarity between its response to extracellular stimuli to that of JNK. JNKK, however, does not activate the ERKs and is unresponsive to Raf-1. Hence, MEKK and JNKK form a protein kinase cascade that leads to JNK activation. This cascade, which stimulates c-Jun transcriptional activity, is distinct from the one involving Raf, MEK, and ERK.

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Both JNK1 and p38 complement an osmosensitive yeast mutant defective in the MAPK homolog, HOG1 (*7*, *8*), suggesting that a mammalian homolog of PBS2, the MAPKK that activates HOG1 (*12*) and can also activate JNK1 (*8*), is their physiologic activator. Because MEK1 and MEK2 do not activate JNK (*11*), we searched for new mammalian MAPKKs that exhibit this function. Vertebrate complementary DNA (cDNA) clones encoding potential MAPKKs—XMEK2 from *Xenopus* and its mouse homolog, MMA1-SEK1—have been isolated (*13*). We isolated and determined the sequence of their human homolog (*14*) (Fig. 1A), and a clone encoding a truncated version of this protein was isolated by Dérijard *et al.* (*15*). The three vertebrate MAPKK-like proteins are very similar to each other (>95% amino acid identity within the kinase domains) and are more similar to PBS2 than to other *Saccharomyces cerevisiae* MAPKK homologs (*16*). Both the mouse and human MAPKK-like proteins can partially complement a PBS2 deficiency (Fig. 1B) (*17*). Transformation with vectors encoding the mammalian MAPKK sequences allowed *pbs2*$^-$ cells to grow in the presence of 0.9 M NaCl, albeit somewhat more slowly than *PBS2*$^+$ cells. Neither clone complemented a HOG1 deficiency or a double deficiency in MKK1 and MKK2 (*17*).

MMA1-SEK1 cDNA was expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein (*18*), purified, and examined for its ability to activate GST-JNK1 in a coupled kinase assay using GST–c-Jun(1–79) (*19*) as a substrate (Fig. 2A). GST-JNK1 alone exhibited little activity,