GPEET procyclin is a major glycosylphosphatidylinositol-anchored protein of procyclic (insect stage) trypanosomes in culture and is heavily phosphorylated in the GPEET pentapeptide repeat. The phosphorylation reaction is a late event and occurs during maturation and transport of GPEET or on the parasite surface by an ecto-protein kinase. Initial biochemical characterization of the GPEET kinase activity now shows that it depends on bivalent cations for maximal activity, is stimulated by sulfhydryl group reagents, and is specific for ATP as phosphoryl donor. No kinase activity is detected in bloodstream form trypanosomes in culture, whereas strong phosphorylation is observed in early procyclic forms. In addition, the GPEET kinase activity is absent from procyclic trypanosomes that have repressed GPEET synthesis but can be induced in these same stocks by conditions, which also induce GPEET expression. However, the presence of an active kinase does not depend on the presence of (functional) GPEET because it can be detected in parasites expressing a non-phosphorylatable GPEET mutant protein and in procyclin null mutant trypanosomes. Interestingly, the presence of the glycosylphosphatidylinositol lipid moiety seems necessary for GPEET to become phosphorylated. Together, the results demonstrate that GPEET and its kinase are expressed during the same life cycle stages and that factors that induce the expression of GPEET in vitro also induce the expression of the GPEET kinase.

Bloodstream forms of the African trypanosome *Trypanosoma brucei* cause both sleeping sickness in humans and nagana in cattle and are transmitted between mammals by the tsetse fly. In the tsetse fly as part of a blood meal, the short stumpy blood-forms rapidly differentiate into procyclic forms in culture and are transmitted between mammals by the tsetse fly. *Trypanosoma brucei* cause both sleeping sickness in humans and nagana in cattle and are transmitted between mammals by the tsetse fly.

This change of surface protein coat must be under tight control; because the closely packed VSG protects the parasite in the mammalian bloodstream against the immune system of the host, a premature loss of VSG would be lethal for the parasite. Similarly, uncontrolled expression of the highly immunogenic procyclin on bloodstream forms would allow the host to mount a strong immune response and eliminate the parasite.

In *T. brucei* procyclin forms the protein surface coat consists of GPEET and GPEET procyclins, both of which are characterized by internal amino acid repeats (reviewed in Ref. 3) and are attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor (4–7). The closely related EP procyclin isoforms are encoded by 3 classes of genes and contain up to 30 tandem repeat units of Glu-Pro, whereas GPEET procyclin is encoded by a single copy gene and contains 5 or 6 pentapeptide (Gly-Pro-Glu-Glu-Thr) repeats (8). The mature form of GPEET is expressed as a non-glycosylated 24–32-kDa protein that is modified on the GPI anchor by a large carbohydrate side chain consisting of branched poly-N-acetyllactosamine groups capped with sialic acid (7). In addition, GPEET is heavily phosphorylated on the threonine residues of its pentapeptide repeats (6, 9, 10) making it a highly negatively charged surface protein.

The relative amounts of EP and GPEET can vary considerably in culture, depending on the trypanosome stock and the culture medium. In one stock, *T. brucei* procyclic forms express almost exclusively EP on their surface (11), whereas in another stock GPEET represents >85% of the procyclins (6). During synchronous differentiation of the pleomorphic strain AnTat 1.1 from bloodstream forms to procyclic forms in DTM medium in vitro, EP and GPEET are co-induced (9, 12) and continuously expressed during culture in this medium (12). A similar co-induction of EP and GPEET expression is also observed during differentiation of stumpy forms in SDM-79 medium. In contrast however, after 10 days of culture in the same medium, procyclic forms completely repress surface expression of GPEET, whereas EP expression is maintained. This down-regulation of GPEET can be prevented by the addition of glycercol to SDM-79 indicating that external signals can influence procyclin expression (12). The regulation of GPEET expression by external signals is also observed in *T. brucei* 29-13 (a derivative of *T. brucei* 427) procyclic forms, which express almost no GPEET in SDM-79 but show dramatic up-regulation of GPEET under conditions of glucose depletion (11). In contrast to the glycerol effect in AnTat 1.1, which cannot be reversed by the addition of glycerol to parasites after they have stopped expressing GPEET (12), the glucose-depletion phenotype in 29-13 parasites is fully reversible by increasing the glucose concentration in the medium (11).

The observation that GPEET is phosphorylated (6, 9, 10) is...
surprising because phosphorylation of surface proteins is a rare event in biology, and its functions are largely unknown. Phosphorylated GPEET is detected close to the flagellar pocket and on the surface of *T. brucei* 427 procyclic trypanosomes, suggesting that the phosphorylation reaction occurs late during maturation and transport of GPEET, or on the parasite surface (9). GPEET phosphorylation occurs on six of seven threonine residues in the pentapeptide repeat (10) and can be detected by antibodies that specifically recognize the unphosphorylated (K1 antisera) or phosphorylated (monoclonal antibody (mAb) 5H13) form of the protein (9). Replacement of all threonine residues in the GPEET repeat by alanines results in a GPEEA mutant protein, which can no longer be phosphorylated (13). *T. brucei* 427 parasites expressing GPEEA instead of GPEET are viable in culture and can establish an infection in the tsetse fly midgut (13).

Phosphorylation of surface proteins and extracellular components has also been described in other parasites. Cell membrane-associated and secreted kinases have been shown to phosphorylate parasite or host proteins in *Trichinella spiralis* infective larvae (14, 15), *Toxoplasma gondii* tachyzoites (16), and *Leishmania* spp. promastigotes (17–21). It has been speculated that the ecto-protein kinases from *Leishmania* may be involved in modulating the host immune system or host-parasite interactions. Although some basic characterization of the kinases has been reported, none of the enzymes has been purified to homogeneity, and no information is available at the genetic level.

Surface protein phosphorylation is mediated by ecto-protein kinases acting extracellularly on ecto-domains of cell surface proteins or on soluble proteins using extracellular ATP as a substrate. In particular, ecto-protein kinase activities have been described at the surface of a number of cells in the circulatory system, such as platelets, T lymphocytes, neutrophils, epithelial and endothelial cells (reviewed in Ref. 22), on tumor cells (23–27), and on mast cells (28). In addition, ecto-protein kinases phosphorylation seems to be a widespread phenomenon in neuronal cells and tissue (reviewed in Ref. 29). Ecto-protein kinases show catalytic specificities of known intracellular protein kinases. These include various cAMP-dependent and -independent protein kinases, protein kinase C and protein kinase CK2 (formerly known as casein kinases 2) or CK2-like enzymes (reviewed in Ref. 22). So far only a few of these enzymes have been characterized on the molecular or biochemical level (24, 30–33). Interestingly, two previously identified proteins, CD4 receptor on T helper cells (34) and a 56-kDa protein from group C streptococci (35), were reported to display ecto-protein kinase activity.

To characterize the kinase responsible for GPEET phosphorylation, we performed a basic biochemical analysis of the kinase activity using membranes from hypotonymically lysed procyclic trypanosomes. In addition, we studied the expression of the kinase activity in procyclic forms expressing mutant forms of GPEET and in trypanosomes from different life cycle stages expressing no or high levels of GPEET.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise stated, all reagents were of analytical grade and were from Merck, Sigma, or BDH (Poole, UK; Tris-HCl, glycine, and Triton X-100 were purchased from ICN (Tägerig, Switzerland). The trypanosome culture media SDM-79 (36), DTM (37), and HMI-9 (38) were prepared with products from Invitrogen, Inotech (Dottikon, Switzerland), JRH (Hampshire, UK), and Amimed (BioConcept, Allschwil, Switzerland). Fetal bovine serum (FBS) and dialyzed inositol, was done essentially as described elsewhere (45). Briefly, purified, [H]-labeled, and dephosphorylated GPEET was incubated overnight at room temperature in 200 μl of 200 mM sodium acetate, pH 4.0, containing 250 mM NaNO₂ and 0.02% (v/v) Triton X-100. Control sample was incubated in 200 μl of buffer containing 250 mM NaCl instead of NaNO₂. The reaction was stopped by the addition of 5 μl of 6 M HCl, and the products were separated by octyl-Sepharose chromatography as mentioned above. The water-soluble cleavage products eluted in the flow-through of the column, whereas uncleaved GPEET bound to the column material and was eluted by the application of 0.5 M NaCl. The elution of the pentapeptide control sample was incubated in 200 μl of buffer containing 250 mM NaCl instead of NaNO₂. The reaction was stopped by the addition of 5 μl of 6 M HCl, and the products were separated by octyl-Sepharose chromatography as mentioned above. The water-soluble cleavage products eluted in the flow-through of the column, whereas uncleaved GPEET bound to the column material and was eluted by the application of 0.5 M NaCl. The elution of the pentapeptide

**Phosphorylation Assay**—In the standard phosphorylation assay, membranes from 2 × 10⁷ trypanosomes were incubated in 60 μl of 10 mM Tris-HCl, pH 7.4, containing 1 mM MgCl₂, 280 μM MgCl₂, 0.02% (v/v) Triton X-100, and 8.3 mM 32P-labelled chloromercuribenzoate exactly as described before (44). In some experiments, purified dephosphorylated GPEET from 2 to 5 × 10⁷ trypanosomes was added as exogenous substrate. The phosphorylation...
The enzymatic properties of the GPEET kinase were studied using a crude membrane preparation of T. brucei procyclin forms. Parasites were lysed in hypotonic buffer followed by repetitive passage through a narrow gauge syringe, and the thoroughly washed membranes were used to phosphorylate endogenous GPEET. Initial studies showed that the phosphorylation reaction is dependent on bivalent cations with maximal activity at 1 mM CaCl₂ and 280 μM MgCl₂, and complete inhibition in the presence of 5 mM EDTA or 5 mM EGTA (results not shown). In some experiments, purified GPEET was added to the membranes as an exogenous substrate. To ensure proper inhibition of GPEET in the incubation mixture, and to allow a direct comparison between individual experiments, 0.02% (w/v) Triton X-100 was routinely added to all reactions. Under these standard conditions, incubation of procyclin membranes in the presence of [γ³²P]ATP resulted in the phosphorylation of multiple bands ranging between 20 and 50 kDa (Fig. 2A, lane 1). Phosphorylation of GPEET (at 24–32 kDa) was only visible after longer exposure of the gel (results not shown). The labeling pattern was similar but not identical to the one obtained using intact parasites (see Fig. 1).

It has been shown previously (49–52) that certain protein kinases are inhibited by the sulfhydryl group reagents, pCMB. Unexpectedly, and in contrast to these reports, we found that the presence of increasing concentrations of pCMB resulted in strong activation of GPEET phosphorylation (Fig. 2A, lanes I–7). At 8.3 mM pCMB, which represents the concentration showing maximal inhibition of an ecto-protein kinase of rabbit peritoneal polymorphonuclear leukocytes (49), GPEET showed maximal phosphorylation (Fig. 2A, lane 7). At concentrations between 10 μM and 1 mM, pCMB inhibited the phosphorylation of proteins at 34 and 48 kDa (the appearance of the 34-kDa band varied between experiments). The effects of higher concentrations of pCMB on GPEET phosphorylation could not be tested due to its limited solubility in aqueous solution. We therefore repeated the experiments using a more soluble, related compound, p-chloromercuriphenylsulfonic
acid (pCMPS), and we found that, like pCMB, it strongly increased phosphorylation of GPEET in a concentration-dependent way (Fig. 2B). Maximal activation was observed at 0.1 mM pCMPS (Fig. 2B, lane 5), whereas at concentrations ≥10 mM, pCMPS completely blocked phosphorylation of all proteins (Fig. 2B, lane 7). Because the two compounds penetrate cell membranes poorly (pCMB (55)), or not at all (pCMPS (54)), they are often used to probe cell surface-oriented sulfhydryl groups. We therefore applied these reagents to study phosphorylation of intact procyclic trypanosomes and found that they induced a similar concentration-dependent increase in GPEET phosphorylation as they did for crude membranes (results not shown), again demonstrating that the reaction takes place on the surface of the parasite.

pCMB and pCMPS are reagents that affect biological activities of proteins via specific chemical modification of sulfhydryl groups (55). To obtain further information about their mode of action in GPEET phosphorylation, we replaced the organic mercurial compounds by two other sulfhydryl group reagents, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) or N-ethylmaleimide in the standard assay. The results showed that the presence of increasing concentrations of 5,5'-dithiobis(2-nitrobenzoic acid) or N-ethylmaleimide (1 μM to 10 mM final concentrations) led to a decrease in phosphorylation of the 48-kDa band and a small increase in GPEET phosphorylation (results not shown). The effect of pCMC could be completely blocked by the presence of 10 mM dithiothreitol in the reaction mixture (result not shown).

To characterize the GPEET kinase further, incorporation of radioactivity into GPEET in procyclic membranes was monitored as a function of incubation time and temperature. We found that in the presence of pCMB, GPEET phosphorylation was detectable already after 1 min of incubation at 27 °C and increased with increasing incubation times (Fig. 3A, lanes 1–7). When membranes were incubated for 15 min, little or no phosphorylation of GPEET was observed at 4 °C, whereas a strong signal was detected at 27 °C (Fig. 3B). Higher temperatures resulted in a decrease in GPEET phosphorylation, and boiling of the membranes for 5 min prior to the assay completely destroyed the activity (results not shown).

Certain kinases such as protein kinase CK2 (formerly known as casein kinase 2), a ubiquitous intracellular serine/threonine kinase that was first reported to phosphorylate β-casein (reviewed in Ref. 56), not only use ATP as phosphoryl donor but also other NTPs. Because several described ecto-protein kinases display characteristics of a protein kinase CK2 (20, 24, 57), we studied the specificity of the GPEET kinase for NTPs by adding excess amounts of non-radioactive ATP, CTP, GTP, or UTP to procyclic membranes incubated in the presence of [γ-32P]ATP (Fig. 4). The results showed that non-radioactive ATP, as expected, inhibited the incorporation of radioactivity into GPEET by competing with the radiolabeled substrate for the phosphorylation sites (Fig. 4A). In addition, weak inhibition of GPEET phosphorylation was observed in the presence of increasing amounts of CTP (Fig. 4B), whereas GTP and UTP showed no effect (Fig. 4, C and D).

**The GPEET Kinase in T. brucei Procyclic Forms Expressing GPEET Mutant Proteins—**To characterize further the GPEET kinase in procyclic trypanosomes, GPEET−/− (T/−) and GPEEN−/− (A/−) membranes were tested for their ability to
were analyzed by SDS-PAGE and autoradiography. Incubation by the addition of sample buffer. Phosphorylated proteins addition of \[ \text{described in Fig. 2. The phosphorylation reaction was started by the} \]

Membranes from 2°/H11003 phosphorylation reaction and incubated with GPEEA/...because the reaction does not go to completion (we typically obtain 70–75% cleavage), we separated cleaved and uncleaved GPEET after nitrous acid treatment, which is known to cleave the covalent linkage between the non-acetylated glucosamine and the inositol moiety to release a hydrophilic protein (45). The extent of anchor cleavage can be determined by measuring the distribution of radioactivity between the aqueous and butanol-rich phases after partitioning of [\(^{3}H\)ethanolamine-labeled GPEET between water and butanol. Because the reaction does not go to completion (we typically obtain 70–75% cleavage), we separated cleaved and uncleaved GPEET after nitrous acid treatment by octyl-Sepharose chromatography. Equal amounts of radioactivity, representing equal amounts of cleaved and uncleaved GPEET, were subsequently used as substrates for the phosphorylation reaction and incubated with GPEEA/ membranes as a source of the kinase. Interestingly, we found that nitrous acid-treated GPEET could no longer be phosphorylated, whereas mock-treated GPEET was readily labeled with \[ \gamma^{32}\text{P} \] ATP (Fig. 6, lanes 1 and 2). Analysis of the two \(^{3}H\)-labeled forms of GPEET by SDS-PAGE followed by fluorography demonstrated that the primary sequence of the protein was not affected by nitrous acid treatment and that similar amounts of protein were used for the phosphorylation reaction (Fig. 6, lanes 3 and 4).

Phosphorylation by Procylic Form Trypanosomes after Modulation of GPEET Expression—It has been shown (12) that the expression of GPEET can be modulated in vitro by the presence or absence of glycerol in the culture medium. In agreement with this report, we found that when procyclic forms of AnTat 1.1 trypanosomes were cultured in SDM-79 medium in the presence of 20 mM glycerol, parasites expressed GPEET as demonstrated by the reactivity of two anti-GPEET antibodies with parasite membranes on immunoblots (Fig. 7A, lanes 1 and 3). In addition, in the absence of glycerol, GPEET expression was completely absent (Fig. 7A, lanes 2 and 4). Accordingly, endogenous GPEET in procyclic forms cultured in the presence of glycerol was labeled with \[^{32}\text{P} \] ATP, whereas no labeled GPEET was detected in parasites cultured in the absence of glycerol (Fig. 7B, lanes 1 and 3). Interestingly, when purified GPEET was added to procyclic form trypanosomes after down-regulation of endogenous GPEET expression, the exogenous substrate was not phosphorylated (Fig. 7B, lane 4). In contrast, control cells expressing endogenous GPEET showed increased phosphorylation in the presence of exogenous GPEET (Fig. 7B, lane 2). These results show that procyclic form parasites, in which GPEET expression is down-regulated by the removal of glycerol from the culture medium, also have no GPEET kinase activity and suggest that the expression of GPEET and its kinase may be linked. To test this hypothesis further, we used procyclic forms of another stock, T. brucei 29-13, which normally does not express GPEET but induces its biosynthesis in response to lowering the glucose concentration in the culture medium (11). As for the AnTat 1.1 strain, the expression of GPEET in 29-13 parasites was tested by immunoblotting using the two antibodies that recognize unphosphorylated (K1 antisera) and phosphorylated (mAb 5H3) GPEET, and confirmed the absence or presence of GPEET in trypanosomes cultured in medium containing normal or low concentrations of glucose,
Fig. 6. Removal of the GPI lipid moiety inhibits GPEET phosphorylation. GPEET from T. brucei 427 procyclic forms, which has been labeled in the GPI moiety using[^3H]ethanolamine, was purified by octyl-Sepharose chromatography, treated with nitrous acid, and rechromatographed to separate cleaved from uncleaved protein. Mock-treated (−) or nitrous acid-treated (+)^[^3H]-labeled GPEET was subsequently analyzed by SDS-PAGE and fluorography (lanes 3 and 4) or incubated in the presence of membranes from 2 × 10^7 GPEEA mutant trypanosomes (A−; see Fig. 5) as source of the GPEET kinase (lanes 1 and 2). The phosphorylation reaction was started by the addition of [γ-[^32P]]ATP and terminated after 15 min of incubation by the addition of sample buffer. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

Phosphorylation by Bloodstream Form Trypanosomes—
GPEET expression is absent in T. brucei bloodstream forms and is only induced after triggering parasites to differentiate to procyclic forms (9, 12, 60). We therefore studied the expression of the GPEET kinase in bloodstream forms in culture by labeling them with [γ-[^32P]]ATP in the presence of exogenous GPEET. Because bloodstream form trypanosomes express a surface phosphatase (61), which may interfere with the phosphorylation reaction or immediately dephosphorylate products of an ecto-protein kinase reaction, the experiments were also carried out in the presence of the phosphatase inhibitor NaF (61). Our results show that membranes from bloodstream form trypanosomes were unable to phosphorylate GPEET (Fig. 9), demonstrating that the GPEET kinase is not expressed in this life cycle stage. The presence of NaF in itself had no effect on the kinase because phosphorylation of GPEET in procyclic forms was not affected when the compound was present in the reaction mixture (results not shown).

DISCUSSION

T. brucei procyclic forms express a kinase that phosphorylates GPEET procyclin on multiple threonine residues in the pentapeptide repeats. Initial biochemical characterization of the GPEET kinase activity using crude membranes from procyclic parasites shows that the reaction is dependent on divalent cations and can be stimulated by reagents that bind to free sulfhydryl groups. Our observation that pCMB and pCMPS are potent activators of the GPEET kinase is surprising because both compounds have been described as protein kinase inhibitors (49–52). These organic mercurials bind specifically and stoichiometrically to free sulfhydryl groups and, thus, can be used to determine the number of protein thiol groups (55). Our observation that pCMPS activates the GPEET kinase at concentrations below 1 mm, but inhibits the reaction at higher concentrations, suggests that the kinase has (at least) two different types of sulfhydryl groups. One type is easily accessible to pCMPS (and other sulfhydryl reagents), and its modification results in steric activation of the kinase. The other type of sulfhydryl group only reacts with pCMPS at high concentrations and is involved in catalysis or, alternatively, is essential in maintaining the three-dimensional structure of the enzyme needed for biological activity. Such a dual effect of an organic mercurial on an enzyme has been reported before for the activation and inhibition of cytoplasmic aldehyde reductase by low and high concentrations, respectively, of pCMB (62). Alternatively, the sulfhydryl reagents may affect GPEET phosphorylation by inhibiting a competing phosphatase or by inactivating an inhibitor or a regulatory subunit of the kinase. In addition, we cannot rule out that the mercurials don’t act via blocking protein sulfhydryl groups but by hindering to the enzyme by some other mechanism.

The EFGPEET motif in the GPEET repeats represents a consensus sequence for threonine phosphorylation by protein kinase CK2 (underlined residues are essential for activity and those in italic stimulate phosphorylation (56)). This, together with the observation that protein kinases CK2 or protein kinase CK2-like activities have been reported to be involved in the phosphorylation of a number of surface proteins (see Refs. 21, 24, and 28, for example), prompted us to study some of the typical characteristics of these enzymes with regard to GPEET phosphorylation. We found that, unlike protein kinases CK2, the GPEET kinase uses ATP but not GTP as phosphoryl donor, is not inhibited by low concentrations of heparin, and does not phosphorylate the typical CK2 substrate phosvitin (results not shown). In addition, although a recent report (63) demonstrated the presence of the gene for the protein kinase CK2 α subunit in T. brucei, we can exclude its involvement in GPEET phosphorylation based on its nuclear localization. Together, these results demonstrate that the enzyme responsible for GPEET phosphorylation in procyclic trypanosomes is not related to previously characterized ecto-protein kinases and represents a novel type of enzyme.

Our findings using different life cycle stages of T. brucei and various strains show that the expression of the GPEET kinase correlates with the expression of GPEET. The kinase activity is absent in T. brucei bloodstream forms in culture, whereas strong phosphorylation is observed in T. brucei 427 procyclic forms that express GPEET as their major form of procyclin (6). In addition, the GPEET kinase is present in T. brucei AnTat 1.1 procyclic forms cultured in DTM or SDM-79 medium containing glycerol. Under these conditions, the parasites express high levels of GPEET (12). In contrast, when GPEET expression is down-regulated by removing glycerol from the culture medium (12), the GPEET kinase activity is also repressed. A similar correlation between GPEET and GPEET kinase expression is also observed in T. brucei 29-13 procyclic forms. This strain expresses little or no GPEET under normal culture conditions (11) and shows no GPEET kinase activity. In contrast, after induction of GPEET expression by lowering the glucose concentration in the culture medium (11), GPEET and the GPEET kinase activity are co-induced. Interestingly, however, the presence of an active kinase does not depend on the presence of (functional) GPEET on the parasite surface. Procyclin null mutants lacking all EP and GPEET genes show normal levels of GPEET kinase activity by using exogenously added substrate. In addition, phosphorylation of exogenous
GPEET is also observed in trypanosomes expressing a mutant GPEEA procyclin, which cannot be phosphorylated due to amino acid mutations in the phosphorylation sites (13). Together, these results clearly demonstrate that an active GPEET kinase is expressed during the same life cycle stages as GPEET and that factors which induce the expression of GPEET in vitro also induce the expression of the GPEET kinase.

**FIG. 7.** GPEET and the GPEET kinase activity are co-expressed. A and C, AnTat 1.1 procyclic forms cultured in the presence (+) or absence (−) of 20 mM glycerol (A) or 29-13 procyclic forms cultured at high (9.8 mM; lanes 1 and 3) or low (0.25 mM; lanes 2 and 4) glucose concentrations (C) were analyzed for GPEET expression by SDS-PAGE followed by immunoblotting using mAb 5H3 (lanes 1 and 2) or K1 antiserum (lanes 3 and 4). B and D, membranes from 2 × 10⁷ AnTat 1.1 (B) or 29-13 (D) procyclic forms cultured as in A and C, respectively, were pre-equilibrated at 27 °C in phosphorylation buffer containing 8.3 mM pCMB as described in Fig. 2. The phosphorylation reaction was carried out in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of exogenous GPEET and started by the addition of [γ-³²P]ATP and terminated after 15 min of incubation by the addition of sample buffer. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

**FIG. 8.** GPEET kinase activity in procyclin null mutants. Membranes from 2 × 10⁷ T. brucei AnTat 1.1 procyclin null mutants were pre-equilibrated at 27 °C in phosphorylation buffer containing 8.3 mM pCMB as described in Fig. 2. The phosphorylation reaction was carried out in the absence (−) or presence (+) of exogenous GPEET and started by the addition of [γ-³²P]ATP and terminated after 15 min of incubation by the addition of sample buffer. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.

**FIG. 9.** The GPEET kinase activity is absent from bloodstream form trypanosomes. Membranes from 2 × 10⁷ T. brucei MiTat 1.2 bloodstream forms were pre-equilibrated at 37 °C in phosphorylation buffer containing 8.3 mM pCMB as described in Fig. 2. The phosphorylation reaction was carried out in the absence (−) or presence (+) of exogenous GPEET and/or 1 mM NaF and started by the addition of [γ-³²P]ATP and terminated after 15 min of incubation by the addition of sample buffer. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography.
GPEET Procyolin and Its Kinase Are Co-expressed in T. brucei

The enzymatic or chemical removal of a GPI lipid moiety can generate a soluble protein, which shows major alterations in its biological or physical properties compared with the membrane-associated form. Treatment of GPI-anchored proteins with specific phospholipases or chemical agents has been found to affect enzyme activities (64–66) and ligand- or antibody-binding properties (59, 67, 68), most likely by inducing conformational changes in the proteins (69, 70). Our experiments using chemically modified GPEET revealed a similar phenomenon. The soluble form of GPEET, which was generated by removing the lipid moiety of the GPEET GPI anchor, could no longer be phosphorylated by the GPEET kinase. Because GPEET has been shown before to react very sensitively to the removal of the GPI lipid portion by altering its antibody-binding characteristics (59), the inability to phosphorylate soluble GPEET may be related to conformational changes that occur during GPI lipid removal. Alternatively, the loss of hydrophobicity may affect the interaction of GPEET with the kinase and thereby prevent the phosphorylation reaction. It is possible that nitrous acid also deaminates ω- and ε-amino groups in the GPEET polypeptide chain. However, our observation that prolyl trypanosomases expressing an N-terminally truncated GPEET polypeptide chain. However, our observation that prolyl trypanosomases expressing an N-terminally truncated GPEET polypeptide chain. However, our observation that prolyl trypanosomases expressing an N-terminally truncated GPEET polypeptide chain.