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Evidence for the induction of casein kinase II in bovine lymphocytes transformed by the intracellular protozoan parasite *Theileria parva*

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Theileria parva **is an obligate, intracellular, parasitic protozoan that causes East Coast fever, an acute leukemia-like disease of cattle. *T.parva* and the related parasite, *Theileria annulata*, are unique among protozoa in that their intralymphocytic stages induce transformation of bovid lymphocytes. Comparison of *in vitro* protein kinase activities between uninfected IL-2-dependent T lymphoblasts and *T.parva*-infected lymphocytes revealed a 4.7- to 12-fold increase in total phosphorylation and the induction of a group of *Theileria* infection-specific phosphoproteins. The enzyme that phosphorylates these substrates is a serine/threonine kinase with substrate and effector specificities of casein kinase (CK) II. Northern blot analyses revealed a 3.9- to 6.0-fold increase in CKIIα mRNA in the infected cells relative to the controls. Furthermore, a marked increase of CKII antigen was observed on Western blots of materials prepared from the infected cell lines. The anti-bovine CKII antibody used in these studies immunoprecipitated a protein kinase that phosphorylated casein in a reaction that was inhibited by low (nM) quantities of heparin. Our data show marked increases of bovine CKII at the transcriptional, translational and functional levels in *T.parva*-infected lymphocytes, relative to quiescent cells or IL-2-dependent parental lymphoblasts. Bovine CKII thus appears to be constitutively activated in these cells and we propose that this kinase may be an important element in the signal-transducing pathways activated by *Theileria* in bovid lymphocytes and perhaps in some leukemic cells.**

Key words: casein kinase II/induction/lymphocyte-transformation/*Theileria parva*

Introduction

Casein kinase (CK) II is an operational classification for a serine/threonine-specific and messenger-independent protein kinase, which *in vitro* preferentially phosphorylates acidic protein substrates typified by casein and phosvitin. CKII is a multifunctional enzyme with a ubiquitous distribution in a wide variety of eukaryotic tissues (reviewed by Edelman *et al.*, 1987; Krebs *et al.*, 1988; Pinna, 1990; Tuazon and Traugh, 1991). CKII has been found in the cytosol and in various subcellular compartments (Hathaway and Traugh, 1983; Singh and Huang, 1985; Damuni and Reed, 1988). While recent immunohistochemical evidence suggests that the localization of CKII varies according to the stage of the cell cycle (Filhol *et al.*, 1990; Yu *et al.*, 1991), other data suggest a predominantly nuclear localization throughout the cell cycle (Krek *et al.*, 1992). Because of its potential regulatory roles at the transcriptional, translational and post-translational levels, it has been proposed that CKII functions as a universal modulator of cellular metabolism (Hathaway and Traugh, 1982).

The structure of CKII is usually that of a heterotetramer with possible holoenzyme compositions of $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ or $\alpha'_2\beta_2$ (Hathaway and Traugh, 1982; Dahmus *et al.*, 1984). Several exceptions to this holoenzyme configuration have been reported and include *Drosophila* (Saxena *et al.*, 1987), *T.parva* (ole-MoiYoi *et al.*, 1992) and *Zea mays* (Dobrowolska *et al.*, 1992), all of which appear to have only one catalytic subunit. The α and α′ subunits (M_r range 37 000–45 000) have been shown to be catalytic by photoaffinity labelling with ATP analogues and by functional analysis (Hathaway and Traugh, 1982; Cochet and Chambaz, 1983). Recent studies have compared structural and functional characteristics of the holoenzymes from *Caenorhabditis elegans* (Hu and Rubin, 1990) and *Drosophila* (Lin *et al.*, 1991) with those of the respective, purified recombinant CKIIα subunits expressed in *Escherichia coli*. Although the recombinant α-subunits exhibited most of the functional features of the holoenzyme, the phosphorylation of the native substrates of CKII examined was variable (Lin *et al.*, 1991). Recent sequence analyses of cDNA from a variety of higher eukaryotes have confirmed previous immunochemical and protein sequence data (Dahmus *et al.*, 1984; Takio *et al.*, 1987; Litchfield *et al.*, 1990), suggesting that the α and α′ subunits of CKII are products of different, albeit highly identical, genes (Chen-Wu *et al.*, 1988; Meisner *et al.*, 1989; Hu and Rubin, 1990; Lozeman *et al.*, 1990; Padmanabha *et al.*, 1990; Maridor *et al.*, 1991; Yang-Feng *et al.*, 1991). The β subunits (M_r 24 000–28 000), which autophosphorylate *in vitro* upon exposure of the holoenzyme to ATP or GTP, are required for the optimal modulation of the enzyme by basic stimulatory molecules and for the phosphorylation of specific physiological substrates of CKII (Lin *et al.*, 1991). A number of potential intracellular modulators of CKII activity have been identified and include basic compounds such as polyamines, which enhance the activity of the enzyme (Takio *et al.*, 1987; Filhol *et al.*, 1990), and glycosaminoglycans such as heparin, which are inhibitory (Hathaway and Traugh,

1984). Indeed, extreme sensitivity to heparin inhibition is used to distinguish CKII from other protein kinases.

One of the unique functional features of CKII is its utilization of GTP, as well as ATP, as a phosphoryl group donor. The enzyme phosphorylates mostly serine residues located at unique sequence motifs within which a cluster of acidic amino acid residues are located on the immediate C-terminal side of the target serine or threonine residue (for reviews see Krebs *et al.*, 1988; Pinna, 1990; Tuazon and Traugh, 1991). Among the substrates phosphorylated *in vivo* by CKII are proteins that modulate cellular proliferation or those involved in transformation (reviewed by Meisner and Czech, 1991; Tuazon and Traugh, 1991).

Therefore it is of particular interest that moderate increases of CKII activity have been shown to occur upon exposure of cells to steroid hormones, insulin and growth factors (Sommercorn and Krebs, 1987; Sommercorn *et al.*, 1987; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989; Carroll and Marshak, 1989). The activity of CKII is also markedly elevated in the early developmental stages of *C.elegans* (Hu and Rubin, 1990), in chicken embryos (Maridor *et al.*, 1991) and in maturing sea-star oocytes just before germinal vesicle breakdown (Sanghera *et al.*, 1992). Thus CKII, a protein kinase that is highly conserved over large evolutionary distances (Chen-Wu *et al.*, 1988; Padmanabha *et al.*, 1990; ole-MoiYoi *et al.*, 1992), may play important roles in DNA replication, transcription, translation and in cellular responses to growth stimuli.

T.parva is an obligate, intracellular, protozoan parasite transmitted by the tick vector *Rhipicephalus appendiculatus*. *T.parva* causes East Coast fever, an acute and often fatal lymphoproliferative disease of cattle in eastern and central Africa. Another form of theileriosis, affecting cattle and swamp buffalo, is caused by the related parasite *T.annulata* and is endemic in countries around the Mediterranean and in large areas of Asia including India, southern Russia and China. The mammalian-infective sporozoite stage of *Theileria* is injected into tick attachment sites during feeding. The sporozoites invade host lymphocytes by a receptor-mediated-like endocytotic process, and within minutes of entry the host cell plasma membrane surrounding the sporozoite undergoes rapid dissolution concurrent with the release of sporozoite microneme contents (Fawcett *et al.*, 1982). Within the lymphocyte, the sporozoite differentiates into a multinucleate body, the schizont, which undergoes nuclear division ahead of the host cell due to a shortened or absent, G_2 phase. This process ensures the transfer of the parasite into each daughter cell. The schizont further differentiates into merozoites, which bud-off from the schizont, induce lysis of the lymphocyte and then enter erythrocytes, within which they develop into piroplasms, which are the tick-infective stage of *Theileria*.

T.parva and *T.annulata* are unique among protozoa in that the intralymphocytic schizont of these parasites induces blastogenesis and clonal expansion of bovine T and B cells, respectively. The infected lymphocytes can be propagated indefinitely *in vitro* (Brown, 1983). These cells are considered transformed because they have short generation times *in vitro*, exhibit cellular pleiomorphism and surface phenotype alterations, as detected using monoclonal antibodies, which recognize a variety of leukocyte differentiation antigens or bovine CD analogues (Baldwin *et al.*, 1988). *Theileria*-infected lymphocytes also infiltrate tissues, upon injection into athymic (Irvin *et al.*, 1975) or

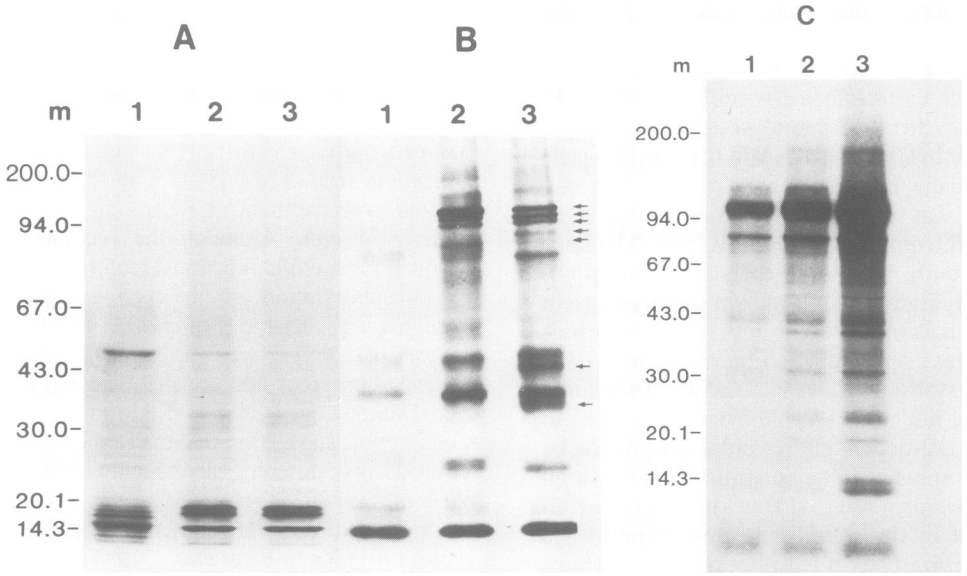

Fig. 1. Kinase assays of the 100 000 *g* particulate fractions from uninfected and *Theileria*-infected bovine lymphocytes. Equal amounts of protein from the 100 000 *g* particulate fractions were incubated with [γ-^{32}P]ATP *in vitro*. The phosphorylated proteins were resolved on 7.5–15% SDS–polyacrylamide gradient gels. **A**. The Coomassie blue-stained gel containing particulate fractions from a cloned uninfected IL-2-dependent bovine cell line G6, the same cell clone (G6-TpM) infected with *T.parva* (Muguga) and from an uncloned cell line B435-TpM (infected with the same parasite stock), is shown (lanes 1, 2 and 3, respectively). The positions of migration of standard markers that were either cold or ^{14}C-labelled are shown in kDa. **B**. The dried gel depicted in panel A was exposed at −80°C with an intensifying screen for 24 h and the autoradiogram is shown. The five arrows (80–116 kDa) show the positions of a group of *Theileria* infection-associated phosphorylated proteins. The arrows at 42 and 33 kDa show two phosphoproteins present in larger amounts in the uncloned *T.parva*-infected B435-TpM cell line. **C**. The 100 000 *g* particulate fractions from the G6-TpM cell line were also incubated using [γ-^{32}P]GTP, rather than [γ-^{32}P]ATP, as phosphoryl group donor. The reaction mixtures were resolved on SDS–PAGE and an autoradiogram of the dried gel is shown. Lanes 1–3 contained 0.1, 0.2 or 0.4 μCi of labelled trinucleotide per mM of GTP, respectively.

severe combined immunodeficient (SCID) mice (Fell *et al.*, 1990), and form tumour-like masses in most organs. We have been studying the molecular mechanisms that may be engaged by the *Theileria* schizonts to transform bovine lymphocytes and have demonstrated a marked increase of a serine/threonine kinase with specificities of CKII in the infected cells. In this report we present evidence for the induction of bovine CKII in *T.parva*-infected lymphocytes at the transcriptional, translational and functional levels.

Results

Enhanced phosphorylation of endogenous protein substrates in Theileria-infected lymphocytes

We examined the protein kinase activity in the 100 000 *g* particulate and supernatant fractions prepared from cloned and uncloned bovine lymphocytes, before and after infection with various isolates of *T.parva*. Incubation of these fractions with [γ-^{32}P]ATP in protein kinase assays, revealed that the incorporation of ^{32}P into endogenous protein substrates was predominantly in the 100 000 *g* pellets. However, when compared with the uninfected cells, the particulate preparations from the *T.parva*-infected cells had a 4.7- to 12-fold higher level of ^{32}P incorporation into protein. Proteins prepared from an uninfected, IL-2-dependent T cell clone G6 (B657.G6), from the same lymphocyte clone after the cell line had been stably infected with sporozoites of *T.parva*, G6-TpM (B657.G6-TpM), and from an uncloned cell line (B435-TpM), that was infected with the same *T.parva* stock, were resolved on SDS−PAGE (Figure 1). The Coomassie blue-stained gel (Figure 1A) did not reveal any significant differences in protein profiles other than a protein band of 47 kDa, which was at times prominent in the uninfected G6 clone (lane 1), but appeared diminished in both cloned (lane 2) and uncloned-infected (lane 3) cell lines. The autoradiogram of the dried gel (Figure 1B) showed several phosphorylated proteins, notably those of 12.5/13.6, 25, 38, 46 and 76 kDa. These proteins were present in variable quantities in all three cell lines. The most dramatic difference between the uninfected, IL-2-dependent G6 clone and both of the *T.parva*-infected cell lines was the presence of three or sometimes five intensely phosphorylated novel protein bands of 80, 86, 92, 96 and 116 kDa (Figure 1B, group of arrows). When [γ-^{32}P]GTP rather than [γ^{32}P]ATP was used as a phosphoryl group donor, the results were almost identical to those shown in Figure 1B, the major difference being less phosphorylation of substrates smaller than 70 kDa (Figure 1C). Exposure of the dried gel for as long as 10 days failed to reveal any phosphorylated proteins in the range of 80−116 kDa in particulate fractions from the uninfected G6 and T19.4 IL-2-dependent cell lines. The infection-associated phosphorylated proteins of 80−116 kDa were also seen in the 100 000 *g* particulate fractions prepared from four additional cell lines infected with

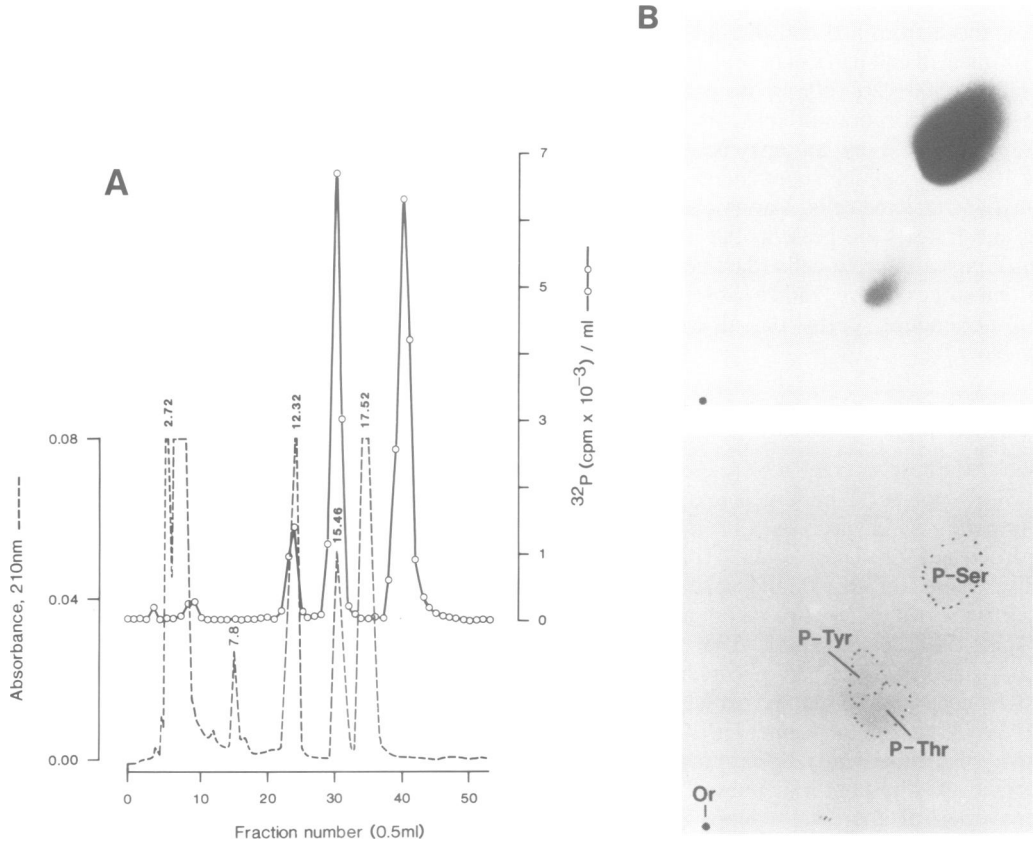

Fig. 2. Phosphoamino acid analysis of residues recovered from *Theileria* infection-associated proteins. Protein bands (80−116 kDa) labelled with ^{32}P were excised from preparative SDS−polyacrylamide gels, the proteins electroeluted, dialyzed and subjected to partial acid hydrolysis. **A**. The hydrolysates were analysed on an Ultrasil Ax HPLC ion exchange column run at 1 ml/min in the presence of phosphoaminoacid internal standards. The lower tracing (----) shows absorbance (210 nm) and retention times (min) for tyrosine (2.72), phosphothreonine (12.32), phosphoserine (15.46) and phosphotyrosine (17.52). The upper panel (-○-○-) depicts ^{32}P counts in fractions (0.5 ml) collected from the column. Orthophosphate eluted at 20.5 min. The partial acid hydrolysate had peaks that co-eluted with phosphothreonine and phosphoserine. **B**. A duplicate sample was analysed by 2D electrophoresis. The migration positions of the ninhydrin-stained internal standards (P-Thr, P-Ser and P-Tyr) are shown.

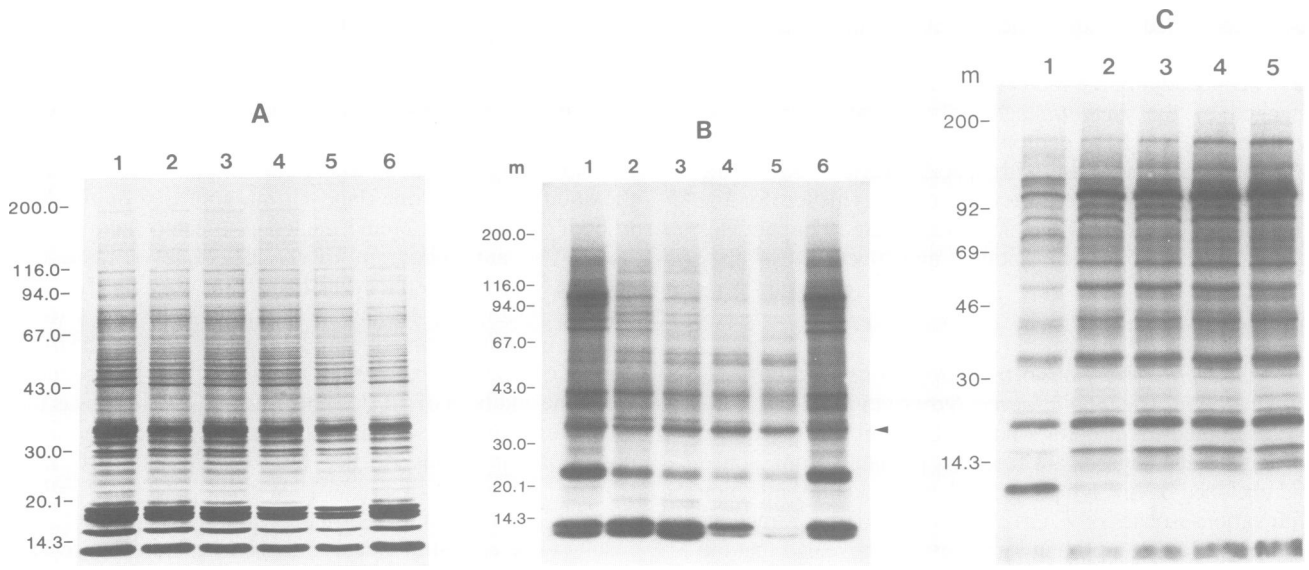

Fig. 3. Modulation of the phosphorylation of *Theileria* infection-associated proteins by heparin and spermine. **A**. The 100 000 *g* particulate fractions prepared from the B435-TpM cell line were employed as a source of kinase and endogenous substrates to assess the effect of heparin (lanes 2–5) on phosphorylation *in vitro*. The proteins were resolved on SDS–PAGE and a Coomassie blue-stained gel is shown. **B**. The autoradiogram of the dried gel shows control samples without heparin (lanes 1 and 6). The effect of heparin on phosphorylation, at concentrations of 2.5, 7.5, 15 and 25 μg/ml, is shown in lanes 2–5, respectively. The arrowhead shows the 32 kDa protein whose phosphorylation appears resistant to inhibition by heparin. **C**. Enhancement of phosphorylation of endogenous substrates by spermine. The *in vitro* kinase assays, followed by analysis on SDS–PAGE, were performed using the 100 000 *g* particulate fractions prepared from the B435-TpM cells in the presence of increasing amounts of spermine. The Coomassie blue-stained gel, similar to Figure 3A, showed identical patterns for all spermine concentrations used. The autoradiogram of the dried gel, in which samples containing 0, 1, 2.5, 5 and 7.5 mM spermine (lanes 1–5, respectively) had been resolved, is shown.

T.parva, as well as those from a B cell line infected with *T.annulata* (Tova). Infected cell particulate fractions, which at dilutions of 1/250–500 (20–60 ng of protein) had undetectable phosphate incorporation into the infection-specific proteins, were used as an enzyme source to phosphorylate the 100 000 *g* cellular supernatants from both uninfected and *Theileria*-infected cells. Variable amounts of these endogenous substrates were present, but only in the supernatants from *T.parva*-infected cells (data not shown). Therefore these substrates were either absent in the uninfected, IL-2-dependent cells or already fully phosphorylated *in vivo*.

Phosphoamino acid analysis of the Theileria infection-associated proteins

The infection-associated phosphoproteins were electroeluted from preparative SDS–polyacrylamide gels and subjected to either alkaline or partial acid hydrolysis. Chromatography of the KOH hydrolysates on Ultrasil Ax HPLC anion exchange columns, yielded mostly P_i and small amounts (1–3%) of radioactivity, which co-chromatographed with the phosphothreonine internal standard. There was no radioactivity in association with either phosphoserine or phosphotyrosine (data not shown). On the other hand, partial acid hydrolysates analysed on the same HPLC column revealed radioactivity that consistently co-chromatographed with phosphothreonine and phosphoserine internal standards (Figure 2A). Recoveries of the total counts from acid hydrolysates, subjected to chromatography on these columns, ranged from 72–85% and comprised ~9% phosphothreonine, 41% phosphoserine and 50% P_i. The identity of phosphoserine and phosphothreonine was further substantiated on thin layer electrophoresis in which the peaks recovered from HPLC co-migrated with their respective ninhydrin-stained internal standards (Figure 2B). No radioactivity was associated with the phosphotyrosine ninhydrin spot.

Modulation of phosphorylation by heparin and polyamines

We have shown that purified *T.parva* schizonts have a protein kinase whose phosphorylation of exogenous substrates is inhibited by heparin (ole-MoiYoi *et al.*, 1992). This observation prompted us to examine the effects of this glycosaminoglycan, other inhibitors and polyamines, on the phosphorylation of endogenous substrates from *Theileria*-infected lymphocytes. The 100 000 *g* particulate fractions from either the G6-TpM or the B345-TpM cell lines were incubated with [γ-^{32}P]ATP and the ^{32}P-labelled proteins analysed on SDS–PAGE. The Coomassie-blue stained gel (Figure 3A) showed about equal amounts of protein in each lane. Interestingly, we noted that increasing amounts of heparin appeared to improve the resolution, particularly of low molecular weight bands. A dose-dependent inhibition of the phosphorylation of the infection-associated family of proteins was revealed by increasing the amount of heparin in the reaction mixtures from 2.5–25 μg/ml (Figure 3B). The degree of inhibition did not appear to correlate with the relative extent of phosphorylation in the absence of the inhibitor. The 80 and 96 kDa proteins were the most sensitive, since their phosphorylation was essentially abolished by 7.5 μg of heparin per ml (Figure 3B, lane 3), whereas trace signals were still detectable in association with the 86, 92 and 116 kDa proteins at the highest dose of heparin used (Figure 3B, lane 5). Although heparin appeared to induce a generalized inhibition of kinase activity, phosphorylation of a few endogenous substrates, such as the 32 kDa protein, was consistently refractory to inhibition by

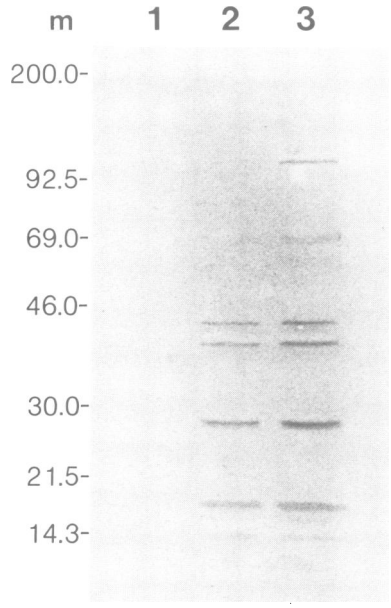

Fig. 4. CKII antigen in *Theileria*-infected cells. The 100 000 *g* particulate fractions prepared from the IL-2-dependent lymphocyte clone G6 (lane 1), the same clone after infection with *T.parva* (G6-TpM; lane 2) or the uncloned cell line B435-TpM (lane 3), infected with the same stock of *T.parva* were resolved on SDS−PAGE, electroblotted and probed with anti-bovine CKII. The Western blots were developed using anti-rabbit IgG conjugated to horseradish peroxidase (Iams *et al.*, 1990).

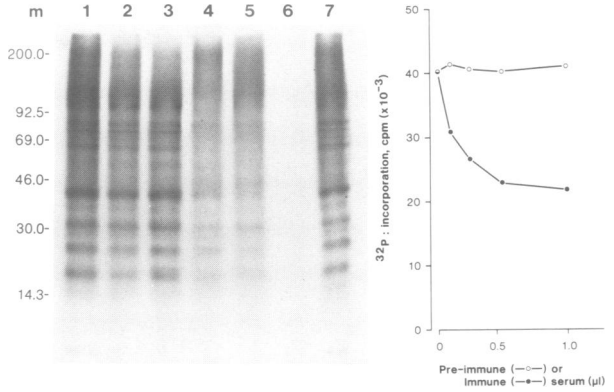

Fig. 5. Inhibition of phosphorylation by rabbit anti-bovine CKII antiserum. Protein kinase assays of particulate fractions prepared from the G6-TpM cell line were performed in the presence of increasing amounts of undiluted antiserum. A sixth of the reaction mixture was precipitated in duplicate with 20% cold TCA−0.1 M sodium pyrophosphate (PP_i), washed twice in 10% TCA and transferred onto Whatman GF/F paper. The paper discs were each washed sequentially in a Minifold apparatus with 10 ml of 10% TCA−0.1 M PP_i, 10 ml 5% TCA−0.1 M PP_i and finally twice with 5 ml acetone. The dried discs were counted in a scintillation counter. Samples, incubated in the presence of normal rabbit serum, were similarly processed. The right hand panel depicts the effect on phosphorylation of 0, 0.1, 0.2, 0.5 or 1 μl of anti-CKII antiserum (-●-●-) or equivalent volume of normal rabbit serum (-○-○-). In each pair of duplicate samples shown the incorporation of ^{32}P into protein varied between 0.7 and 6%. The autoradiogram shows the effect of increasing amounts (0, 0.1, 0.2, 0.5 and 1.0 μl; lanes 1−5, respectively) of anti-bovine CKII antiserum on endogenous substrate phosphorylation. The immune serum (1 μl) had no kinase activity (lane 6) and the normal rabbit serum (1 μl) showed no inhibition of phosphorylation (lane 7).

heparin. It is of interest that the phosphorylation by purified CKII of nucleoplasmin, a 31 kDa protein thought to play an important role in nucleosome assembly, has recently been shown to be resistant to heparin inhibition (Taylor *et al.*, 1987). Of the other CKII inhibitors we examined, pyridoxal 5′-phosphate, Suramin and 2,3-*bis*phosphoglycerate inhibited phosphorylation of the *Theileria*-associated substrates at approximate I_{50} of 800 μM, 50 μM and 4 mM, respectively (results not shown).

The effect of polyamines on the phosphorylation of endogenous substrates by the *Theileria*-associated kinase was studied in the presence of the standard 10 mM Mg^{2+} in the reaction buffer. At a Mg^{2+} concentration of 10 mM, the polyamines putrescine, spermidine and spermine, each at a concentration of 1 mM, stimulated incorporation of ^{32}P into total TCA precipitable substrates and gave 1.1-, 1.35- and 2.0-fold increases over controls, respectively. In such assays, increasing the concentration of spermine in the reaction mixture to 10 or 20 mM showed no further enhancement of phosphorylation, but caused progressive reversal to the basal level found in the absence of the polyamine. The effect of 1−7.5 mM spermine on phosphorylation was also analysed on SDS−PAGE (Figure 3C). The autoradiogram of the gel (Figure 3C, lanes 1−5) showed that spermine stimulated phosphorylation of the majority of endogenous protein substrates. However, the phosphorylation of the peptide doublet of 12.5/13.6 kDa (Figure 3C, lane 3) was a notable exception, since it was essentially abolished by 1 mM spermine (Figure 3C, lane 4). The proteins whose phosphorylation was most dramatically stimulated (2- to 5.2-fold) by spermine included the *Theileria* infection-specific proteins of 86, 96 and 116 kDa.

Increased CKII antigen in Theileria-infected lymphocytes

The data presented above suggested the activation of CKII in *T.parva*-infected lymphocytes. In order to determine the level of CKII antigen in these cells, we used a purified anti-CKII IgG (Dahmus *et al.*, 1984) on Western blots of the 100 000 *g* particulate fractions prepared from the G6 IL-2-dependent lymphoblast clone and from the *T.parva*-infected G6-TpM and B435-TpM cell lines (Figure 4). The immunoblot shows a substantial increase of the CKII in both the G6-TpM and the B435-TpM cell lines infected with *T.parva*. There were no visible bands in the fractions from the IL-2-dependent G6 clone (Figure 4, lane 1), whereas the infected cell lines revealed the typical subunits of bovine CKII; α (43 kDa), α′ (37 kDa) and β (25 kDa) (Figure 4, lanes 2 and 3, respectively). The additional bands (72 and 130 kDa) seen in the fraction from the B435-TpM are unknown antigens that are being further characterized. The three bands (12.5−18.0 kDa) have only been seen in *Theileria*-infected lymphocytes and we speculate that they may well represent cross-reactive antigens associated with the schizonts of *T.parva* CKII (ole-MoiYoi *et al.*, 1992).

Anti-bovine CKII antibody inhibits phosphorylation of Theileria infection-associated proteins

Rabbit anti-bovine CKII serum, which had been raised against the purified holoenzyme, inhibits phosphorylation of exogenous substrates (Dahmus *et al.*, 1984). This antiserum was examined for its effect on the kinase activity detected in *T.parva*-infected bovine lymphoytes. The amount of this

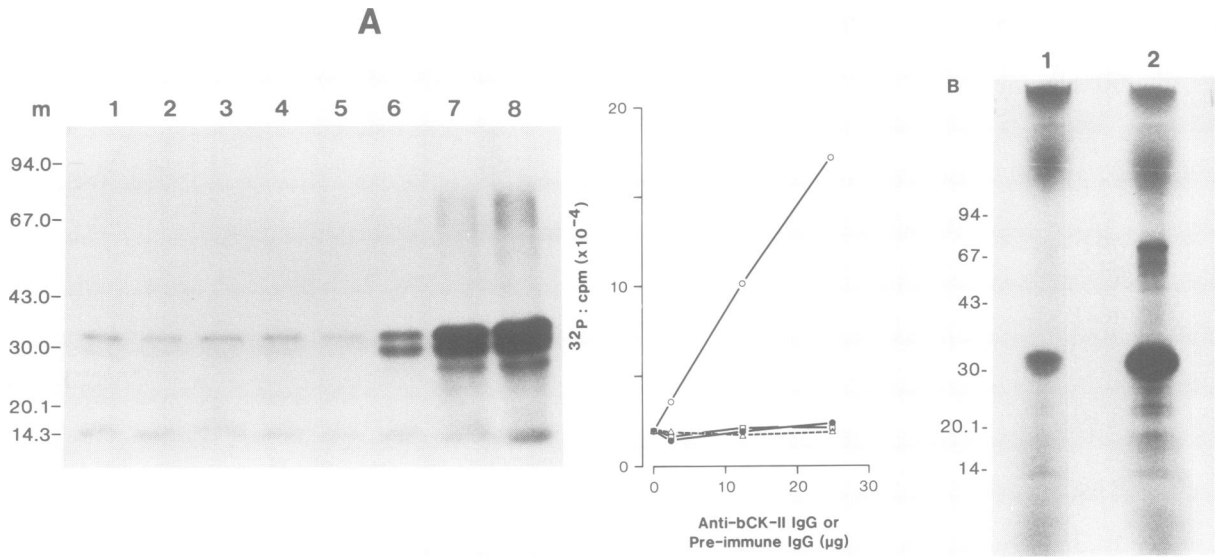

Fig. 6. Immune complex kinase assays. **A**. Protein A−Sepharose was incubated with pre-immune rabbit IgG or anti-bovine CKII IgG. The autoradiogram on the left is that of a dried SDS−polyacrylamide gel in which immune complex kinase assay mixtures, each of which contained 500 μg casein/ml, were resolved. Lanes 1 and 5, no IgG; lanes 2, 3 and 4, 2.5, 12.5 and 25.0 μg of normal rabbit IgG, respectively; lanes 6, 7 and 8, 2.5, 12.5 and 25.0 μg of anti-CKII IgG, respectively. The casein bands shown in the autoradiogram were excised from the dried gel, boiled in 30% H_2O_2 and the ^{32}P was quantified in a scintillation counter. The curves depicted on the right hand panel are those of samples that contained immune IgG (-○-○-) or non-immune IgG (-●-●-) with exogenous casein. The acid precipitable radioactivity in samples without added exogenous substrate, but with the same doses of immune (□) or non-immune (△) IgG, are also shown. **B**. Depicted is an autoradiogram showing the phosphorylation of casein by CKII in the immunoprecipitates (50 μg of anti-CKII IgG) prepared using NP-40-treated 100 000 *g* particulate fractions from the G6 (lane 1) and those from the G6-TpM (lane 2) cell lines.

high titer antiserum was limited, therefore the quantity of the 100 000 *g* particulate fractions assayed were decreased by 75% (5−20 μg protein) and incubated in the kinase reaction with heat-inactivated (60°C, 45 min), undiluted antiserum in doses ranging from 0.1−1.0 μl. Increasing amounts of antiserum revealed progressive inhibition of incorporation of ^{32}P into TCA precipitable protein (Figure 5, right hand panel). The use of 0.1 μl of antiserum in the assay was associated with a 25% decrease in kinase activity. Further increases in the antiserum concentration in the reaction mixture up to 1.0 μl, resulted in only an additional 22−25% inhibition of phosphorylation. Analysis of the ^{32}P-labelled proteins on SDS−PAGE and autoradiography showed a similar decrease in kinase activity with increasing doses of antiserum (Figure 5, left panel). The signal associated with the 116 kDa protein was no longer detectable when 0.5 μl of anti-CKII was used (Figure 5, lane 4). The phosphorylation of the 86 and 92 kDa proteins was diminished by >70% when 1.0 μl of antiserum was used (Figure 5, lane 5). With 1.0 μl of anti-CKII antibody, there was diminution of phosphorylation involving all visualized phosphoproteins (Figure 5, lane 5). The highest concentration of anti-CKII used (1.0 μl) resulted in no detectable endogenous kinase activity (Figure 5, lane 6). Phosphorylation of *Theileria*-infected, bovine lymphocyte 100 000 *g* particulate substrates was not inhibited by 1.0 μl of heat-inactivated, non-immune rabbit antiserum (Figure 5, lane 7 and right panel, upper curve). Thus the inhibition of CKII activity by the immune antiserum was not due to a heat-stable serum factor, but rather to the anti-CKII antibody. Additionally, purified anti-bovine CKII IgG could also only inhibit 75% of kinase activity when used at concentrations as high as 750 μg/ml.

Identification of CKII activity in the immonoprecipitates

Since complete inhibition of kinase activity could not be achieved with this anti-CKII IgG, it was of interest to determine both the specificity of the residual enzymatic activity in immunoprecipitates and to analyse such antigen−antibody complexes for endogenous substrates that might have co-immunoprecipitated with the enzyme. The residual enzymatic activity in the immunoprecipitates of the lysates of *Theileria*-infected lymphocytes (G6-TpM or B435-TpM) was 12- to 18-fold higher than that of the uninfected cells. Figure 6A is representative of results obtained when exogenous casein (500 μg/ml) was included in the kinase reaction; reaction mixtures with no added antibody (Figure 6A, lanes 1 and 5) or those with increasing amounts of normal rabbit IgG (Figure 6A, lanes 2−4), showed no CKII activity. On the other hand, incubation of protein A−Sepharose with 2.5, 12.5 or 25 μg of anti-CKII IgG precipitated enzymatic activity, which readily phosphorylated casein in a dose-dependent manner (Figure 6A, lanes 6, 7 and 8, respectively). The curves shown in Figure 6A (right panel), depict ^{32}P counts recovered from the casein-containing bands excised from the gel. In the absence of exogenous casein, there was no significant acid-precipitable radioactivity (Figure 6A, right panel, lower curves) and no ^{32}P-labelled protein bands were seen on autoradiography of such gels, regardless of whether immune or non-immune IgG was used (data not shown).

We also employed the anti-bovine CKII IgG in immunoprecipitation of kinase activity from the 100 000 *g* particulate fractions prepared from the IL-2-dependent lymphocyte clone G6. Figure 6B depicts the relative amounts of phosphorylation of exogenous casein catalysed by immune

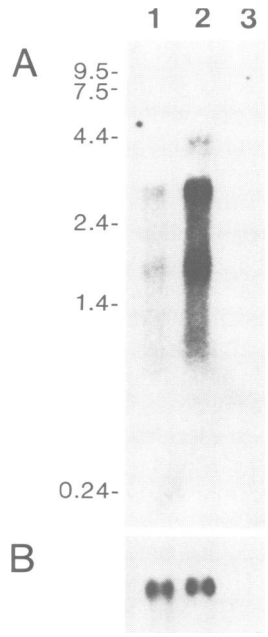

Fig. 7. Increased bovine CKIIα transcripts in the *T.parva*-transformed G6-TpM cell line. **A.** Poly(A)$^+$ RNA isolated from the IL-2-dependent G6 clone (lane 1), its *T.parva*-infected derivative G6-TpM (lane 2) and from *T.parva* piroplasms (lane 3) were separated by formaldehyde−agarose gel electrophoresis, blotted onto nylon filters and hybridized with ^{32}P-labelled bovine CKIIα cDNA (Macklin *et al.*, in preparation; GenBank accession number M93665). **B.** The blot was stripped of radioactivity and reprobed with a cDNA of a constitutively expressed bovine MHC class I gene. The size markers were RNA ladders (BRL) whose lengths are given in kb.

complexes from the G6 cell line (Figure 6B, lane 1) and by those from the infected G6-TpM derivative of this clone (Figure 6B, lane 2). The immune complexes prepared from the infected cell line catalysed the incorporation ^{32}P into casein 12-fold compared with that from the G6 uninfected cell line, in a reaction performed within linearity of the enzymatic activity (Figure 6A, right panel). Although there were faint bands of radioactivity within the molecular weight range of the bovine β subunit of CKII (24−28 kDa; Figure 6A and B) our results neither showed the expected degree of autophosphorylation observed with the β subunit from the uncomplexed holoenzyme nor evidence of any CKII substrates in the immune complexes.

Increased transcript levels of CKIIα in Theileria-infected lymphocytes

To assess the expression of RNA corresponding to bovine CKII in the G6 and G6-TpM cell lines, we employed a bovine CKIIα cDNA (Macklin,M.D., Gobright,E.I. and ole-MoiYoi,O.K., in preparation; GenBank accession number M93665) to probe Northern blots of poly(A)$^+$-enriched RNAs prepared from the G6 and G6-TpM cell lines and *T.parva* piroplasms. The bovine CKIIα probe hybridized with transcripts of 4.3, 3.1 and 1.8 kb in both the RNAs from the uninfected and *T.parva*-infected cell lines (Figure 7A, lanes 1 and 2, respectively). There were no hybridization signals with *T.parva* piroplasm RNA (Figure 7A, lane 3). Based upon densitometric area integration on a Molecular Dynamics Personal Densitometer, there was a 3.9- to 6-fold increase in all three (4.3, 3.1 and 1.8 kb) RNA species from the *T.parva*-infected G6-TpM cell line relative to those from the IL-2-dependent G6 parental clone (Figure 7A, lanes 1 and 2). To determine the amount and to assess the integrity, of the poly(A)$^+$ RNAs analysed in this experiment, the blot depicted in Figure 7A was stripped of radioactivity and reprobed with a cDNA of a constitutively transcribed MHC class I gene (Bensaid *et al.*, 1991). Hybridization of this cDNA gave signals that appear to be of equal intensity (Figure 7B).

Discussion

We have shown that there is a marked increase in protein kinase activity in IL-2-dependent T cells or quiescent lymphocytes after such cells have been infected with the intracellular protozoan parasite *T.parva*. Such an increase in kinase activity, as judged by the incorporation of ^{32}P into TCA-precipitable, total protein or by *in vitro* specific phosphorylation of a group of substrates of 80−116 kDa, is a feature characteristic of all six T lymphocyte cell lines transformed by *T.parva*, as well as of the one B cell line infected with *T.annulata* (Tova) that we have examined. The enzyme that phosphorylated these infection-specific proteins was serine/threonine-specific, cyclic nucleotide-independent, Ca^{2+}/calmodulin- and phosphoinositide-insensitive. The substrate specificity of this phosphotransferase and its susceptibility to modulation by a variety of effectors, including activation by polyamines and inhibition by glycosaminoglycans and other polyanions, strongly suggested that the activity was that of CKII. Although protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinases were detectable in bovine lymphocytes before and after infection with *T.parva*, the former was higher (60%) in the uninfected, IL-2-dependent parental clones (data not shown). This observation is in agreement with that of Carroll *et al.* (1988), who found a marked diminution of PKA, which was concomitant with CKII activation in the early phases of stimulation of growth-arrested W138 cells with serum and in baby rat kidney cells infected with adenovirus. The activity of Ca^{2+}/calmodulin-dependent protein kinase, including the profiles of Ca^{2+}/[^{125}I]calmodulin-binding proteins, was the same for the uninfected and *T.parva*-infected cell lines (data not shown). Other studies of *Theileria*-transformed lymphocytes have revealed an up-regulation of the IL-2 receptor (Dobbelaere *et al.*, 1988), constitutively high levels of NF-κB (Ivanov *et al.*, 1989) and an increase in protein kinase activities, which are still being characterized (Dyer *et al.*, 1992).

The dramatic increase in CKII activity in *T.parva*-transformed cells is supported by the inhibition of enzymatic activity by an anti-bovine CKII IgG, an observation that is in agreement with Dahmus *et al.* (1984), who showed inhibition of purified bovine CKII by this antibody. The anti-bovine CKII IgG also recognized antigens on Western blots, giving the typical α, α′ and β subunit pattern of mammalian CKII, but only in the 100 000 *g* particulate fractions from the *T.parva*-infected lymphocytes. The IL-2-dependent lymphoblasts had no detectable CKII antigen. The antigens of 12.5−18 kDa have only been seen in *T.parva*-infected cells. Interestingly, the residual kinase activity that is detected in the immunoprecipitates prepared from this partially inhibitory, polyclonal anti-bovine CKII IgG is also that of

CKII, since it phosphorylates casein and phosvitin and is inhibited by nM quantities of heparin. In addition, we have demonstrated a significant increase in CKIIα transcripts on Northern blots of poly(A)$^+$ RNA prepared from the *T.parva*-infected G6-TpM cell line, when compared with the RNA from the G6 IL-2-dependent parental clone. The CKIIα cDNA that we used to probe the Northern blots recognized multiple transcripts in both. Similar observations have been reported by others for CKIIα and α′ subunit transcripts from human (Meisner *et al.*, 1989; Lozman *et al.*, 1990) and chicken (Maridor *et al.*, 1991), as well as for the human CKIIβ (Heller-Harrison *et al.*, 1989). The multiplicity of mRNA may arise from the processing of nascent transcripts, alternative splicing or from the use of more than one polyadenylation signal, as has been reported for the human CKIIβ (Heller-Harrison *et al.*, 1989).

Although the physiological functions of CKII are undefined, the activation of bovine CKII by *Theileria* is of particular interest because of the potential perturbations to cellular homeostasis that may arise upon its aberrant induction. It has been reported that moderate activation of CKII occurs in a variety of cell types in response to stimulation by hormones, growth factors or serum (Sommercorn *et al.*, 1987; Carroll *et al.*, 1988; Klarlund and Czech, 1988; Ackerman and Osheroff, 1989; Carroll and Marshak, 1989; Ackerman *et al.*, 1990). These responses were transitory and generally dependent on the magnitude and duration of each stimulus. These reports are consistent with the hypothesis that CKII may be an important element in growth factor- and hormonally-stimulated signalling pathway(s). Significant elevations of CKII activity have also been reported in virally-transformed cells, in human leukemias and in solid tumours (Münstermann *et al.*, 1989; see reviews by Pinna, 1990; Tuazon and Traugh, 1991). Therefore there has been considerable interest in recent years in identifying and characterizing pathways by which CKII and similar kinases are activated, and how in particular an enzyme such as CKII, which is localized to both the nucleus and cytosol, contributes to signal transduction.

Many substrates for CKII have been identified and include membrane receptors, cytoskeletal proteins, cytosolic proteins modulating metabolic responses and those involved in translational control (see reviews by Pinna, 1990; Tuazon and Traugh, 1991). Among these substrates of CKII are nuclear proteins, some of whose structurally-altered forms are implicated in oncogenic transformation and include Fos (Carroll *et al.*, 1988), Myc (Lüscher *et al.*, 1989), Myb (Lüscher *et al.*, 1990), c-ErbA (Glineur *et al.*, 1989) and the tumour suppressor protein p53 (Meek *et al.*, 1990). In several of these proteins, the loss of CKII phosphorylation sites by truncation or amino acid replacement is associated with oncogenic conversion (see review by Meisner and Czech, 1991). Another important group of substrates for CKII are the transforming proteins from several DNA tumour viruses including the large T antigen of SV40 (Grasser *et al.*, 1988; Krebs *et al.*, 1988), E1A of adenovirus (Carroll *et al.*, 1988) and E7 of human papilloma virus type 16 (Grasser *et al.*, 1988; reviewed by Meisner and Czech, 1991). Several of these proteins have been shown to be phosphorylated *in vivo* at the specific CKII sites, which are located 10–60 amino acid residues from their nuclear localization sequence (NLS) motifs (Rihs *et al.*, 1991). In contradistinction to the effects of CKII phosphorylation site alterations in proto-oncogene products, mutations affecting CKII sites in some of these viral proteins render such proteins transformation-defective (Barbosa *et al.*, 1990).

In addition to its potentially central role as a transducer of signals for growth to the nucleus, CKII may also have important regulatory functions in the cell cycle, since the regulation of its activity appears itself to be cell cycle-dependent. Mullner-Lorillon *et al.* (1988) linked the activation of CKII in mature *Xenopus laevis* oocytes to the prophase/metaphase transition in meiotic cell division. Carroll and Marshak (1989) showed that the recruitment of human W138 lung fibroblasts from quiescence with serum resulted in activation of CKII in a cyclical manner suggesting stimulation of the enzyme during the transitions from G_0 to G_1 and from G_1 to S. More recently Cardenas *et al.* (1992) have shown that the phosphorylation of topoisomerase II from *Saccharomyces cerevisiae* by CKII occurs in *in vivo* and varies with the cell cycle. Additionally, in an attempt to determine the biological functions of CKII in *S.cerevisiae*, Padmanabha *et al.* (1990) showed that mutations of both the catalytic subunits of CKII are lethal, but that the cells could be rescued by introduction of the *Drosophila* CKIIα subunit. Yeast cells bearing such double mutations grow to expected size but fail to divide. Furthermore, a recent report shows that *in vitro*, CKII is phosphorylated on the β subunit by p34^{cdc2} (Mullner-Lorillon *et al.*, 1990). A demonstration of such phosphorylation of CKII *in vivo* would be consistent with the idea that CKII may itself be recruited into the maturation promoting factor (MPF) cascade of the cell cycle. Indeed, that some substrates of p34^{cdc2} (Draetta, 1990) are substrates for CKII, including the cell growth-inhibitory protein p53 and large T antigen of SV40, both of which have been shown to be physically associated with p34^{cdc2} kinase, supports this notion (Meek *et al.*, 1990; Nigro *et al.*, 1992).

The post-receptor pathways involved in growth factor-induced activation of CKII are ill-defined. However, Carroll and Marshak (1989) showed that stimulation of lung fibroblasts with phorbol esters led to activation of CKII, suggesting the involvement of protein kinase C (PKC) in the modulation of the activity of CKII. Indeed, CKII, purified from *Pisaster ochraceus*, is phosphorylated on the β subunit *in vitro* by rat brain PKC, with a resultant enhancement of the phosphotransferase activity of CKII (Sanghera *et al.*, 1992). PKC interacts with other cytoplasmic factors in the integration and transmission of stimuli, which ultimately lead to cellular differentiation or proliferation. One of these is the serine/threonine-specific kinase Raf-1 (Siegel *et al.*, 1991), which is among the proteins phosphorylated by the activated PDGF receptor (Morrison *et al.*, 1989; reviewed by Heldin and Westermark, 1990; Cross and Dexter, 1991). Because Raf and PKC may lie upstream in a pathway leading to CKII activation, we compared levels of these enzymes in uninfected and *T.parva*-transformed lymphocytes, but were unable to detect any differences in antigen levels and functional activity, respectively. For example, an anti-chicken Mil antibody (Dozier *et al.*, 1987) revealed the presence of equal amounts of a broad protein band of 72–74 kDa in infected and uninfected cells (data not shown).

Evidence has been presented showing that exposure of T lymphocytes to mitogenic stimulation (Geahlen and Harrison, 1984) and induction of B cell cycle progression by a phorbol ester and ionomycin (DeBenedette and Snow, 1991) are associated with increases in the enzymatic activity of CKII. In our studies on *T.parva*-induced lymphocyte

transformation, we have found evidence showing induction of bovine CKII. Furthermore, we have identified a *T.parva* CKIIα subunit whose deduced amino acid sequence is 68% identical to that of the bovine and human CKIIα subunits (ole-MoiYoi *et al.*, 1992; Macklin *et al.*, in preparation, GenBank accession number M93665). The *T.parva* CKIIα subunit, in addition to having all the structural features considered unique to the catalytic subunits of CKII, has an N-terminal sequence with structural features of signal peptides (ole-MoiYoi *et al.*, 1992). This is intriguing and raises the possibility that a parasite CKIIα subunit is secreted into the host cytosol or inserted into the parasite plasma membrane at some stage during the cell cycle of *T.parva*. From either of those locations, this remarkably conserved enzyme could potentially alter cell cycle regulation or influence the induction of bovine CKII by engaging a positive autoregulatory pathway, such as has been reported for c-*jun* (Cooper, 1990). Heller-Harrison and Czech (1991) have recently shown a 2- to 5-fold enhancement of endogenous CKII activity, depending on whether one or both subunits had been introduced, upon transfection of human CKIIα or β subunits into COS-1 cells. It had been proposed that constitutive production of one or more critical elements in signal transducing cascades may result in aberrant cellular proliferation (Heldin and Westermark, 1984). The dramatic induction of bovine CKII in all the *Theileria*-transformed lymphocyte cell lines studied may arise as a consequence of parasite-induced deregulation of normal elements controlling cellular proliferation at the post-receptor level. We propose therefore that *Theileria* may cause bovid lymphocyte transformation by modulation of controlling elements in signalling pathways, normally activated by hormones, mitogens or growth factors and which may in turn lead to the induction of casein kinase II.

Materials and methods

Cloning of bovine lymphocytes and infection with T.parva

IL-2-dependent bovine cell lines (B657.G6 and T19.4) were cloned by limiting dilution from Con A-stimulated bovine lymphocytes as previously described by Brown and Grab (1985) and Morrison *et al.* (1987). Characterization of these cell lines with monoclonal antibodies to bovine lymphocyte differentiation antigens have shown that they belong to the $CD8^+$ subset of T cells (Baldwin *et al.*, 1988). These IL-2-dependent cell lines were infected *in vitro* by incubation of cells with theilerial sporozoites purified from dissected salivary glands of infected *R.appendiculatus* ticks according to Dobbelaere *et al.* (1984). A bovine cell line infected with *T.annulata* (Tova) was also used in these studies and has been described by Conrad *et al.* (1987).

Uninfected, IL-2-dependent (B657.G6), as well as *Theileria*-infected lymphoblastoid cell lines, were cultured in RPMI 1640 medium containing 25 mM HEPES (Gibco) supplemented with 10% (v/v) heated-inactivated fetal bovine serum (Hyclone), 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 50 μg gentamycin/ml. The cells were incubated at 37°C in an atmosphere of 3–5% CO_2 in air as previously described by Brown (1983) and Brown and Grab (1985). L-15 medium, which was supplemented with 10% (v/v) tryptose phosphate broth in addition to fetal bovine serum, L-glutamine, 2-mercaptoethanol and gentamycin, as for the RPMI 1640, was used to culture the cell line infected with *T.parva* Muguga (B435-TpM). Altogether we analysed six different T cell lines infected with *T.parva* and one infected with *T.annulata* (Tova). The IL-2-dependent cell clone (B657.G6) and its derivative, infected with *T.Parva*, (B657.G6-TpM) will henceforth be referred to as G6 and G6-TpM, respectively.

Preparation of lymphocyte homogenates and immunoprecipitation

For preparation of homogenates for protein kinase assays, uninfected IL-2-dependent or *Theileria*-infected cells were washed 2–3 times in phosphate buffered saline and suspended in cold homogenization buffer [250 mM sucrose, 25 mM Tris–HCl, 0.01 mM EDTA, 5 mM $MgCl_2$, 120 mM KCl, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 8 μg aprotinin/ml, pH 7.2]. The cells were disrupted at 4°C by a Bronson ultrasonic cell disruptor with a jacket horn for 2 min (0.5 s pulses) at a setting of 230 watts. The homogenates were centrifuged at 100 000 *g* for 30 min at 4°C. The 100 000 *g* particulate or supernatants fractions were recovered, the pellet resuspended in a volume of buffer equal to that of the supernatant and analysed for endogenous protein kinase, as well as for substrates associated with theilerial infections. When it was necessary to analyse the phosphorylation of exogenous substrates or to detect the presence of endogenous substrates in uninfected cells, highly diluted (1/250–1/500; 20–60 ng of protein) 100 000 *g* particulate fractions, which showed minimal amounts of endogenous substrates, were employed as the source of protein kinase.

Protein kinase activity in the 100 000 *g* particulate or supernatant fractions, was routinely assayed for 10–15 min at 23°C in incubation mixtures containing 10 mM $MgCl_2$, 120 mM KCl, 50 mM Tris–HCl (pH 7.2), 1 mM isobutylmethylxanthine (IBMX), 15 μM aprotinin, 40 μM [γ-^{32}P]ATP (0.25–1 μCi/mM) or 40 μM [γ-^{32}P]GTP (0.1–0.4 μCi/mM) and 20–80 μg of protein from the fractionated lysate, in a final volume of 100 μl. Various compounds were tested for their effect on protein kinase activity including: cyclic nucleotides, heparin, 2,3-*bis*phospho glycerate, pyridoxal 5′-phosphate and polyamines (Sigma); or the anti-trypanosomal, polysulfonated naphthylamine, Suramin (Bayer). When the effect of these modulators on phosphorylation was to be tested, the enzyme was added into reaction mixtures containing the inhibitor, the [γ-^{32}P] ATP or [γ-^{32}P] GTP, and the other reaction ingredients. For quantitation of ^{32}P incorporation into protein substrates, quadruplicate 10 μl samples were each added into 1 ml of cold 20% TCA–0.1 M pyrophosphate (PP_i) and processed as described by Hathaway and Traugh (1984). Alternatively 10–20 μl samples were spotted in duplicate onto Whatman P81 ion exchange chromatography paper (Whatman) and extensively washed with 75 mM orthophosphoric acid. The paper discs were dried and ^{32}P-incorporation into acid precipitable material quantified by scintillation counting.

A rabbit anti-bovine CKII serum [a gift from Grace and Michael Dahmus (Dahmus *et al.*, 1984), University of California, Davis] was heat-inactivated (65°C, 45 min) and its effect on phosphorylation of the *Theileria*-associated endogenous protein substrates assessed. The heat-inactivated antiserum was also used as the starting material for purification of anti-CKII IgG. Control IgG was similarly prepared from pre-immune rabbit serum.

Immunoprecipitates were prepared by incubation of equal amounts of immune or non-immune IgG with protein A–Sepharose (Pharmacia) for 2 h at 4°C followed by washing three times in PBS. Settled antibody–resin complexes (25 μl) were suspended in 25 μl of lymphocyte homogenization buffer made 200 mM NaCl and 1% NP-40. The 100 000 *g* particulate fractions from uninfected and *Theileria*-infected cells were suspended in the latter buffer at 4°C, extensively vortexed and then centrifuged at 100 000 *g* for 30 min. The supernatants (50 μl) were incubated (2 h at 4°C) with the antibody–resin complexes, followed by washing four times in 1 ml aliquots of Dulbecco's PBS (pH 7.4) in 0.1% NP-40. The immune complexes were washed twice in the kinase reaction buffer and resuspended in 25 μl of the same buffer. Immune complex kinase assays were performed, with and without exogenous casein (0.5 to 2 mg/ml) and the ^{32}P incorporation was quantified by H_3PO_4 precipitation and analysed on SDS–PAGE.

RNA isolation and Northern blot analysis

Preparations of poly(A)$^+$-enriched RNA were made from the IL-2-dependent cell lines, *T.parva*-infected lymphocytes and from *T.parva* piroplasms, and analysed on formaldehyde denaturing gels, as previously described by ole-MoiYoi *et al.* (1992). A bovine CKIIα cDNA (Macklin *et al.*, in preparation; GenBank Accession Number M93665) was used as a hybridization probe on these Northern blots.

Analytical methods

Samples containing equal amounts of protein from *T.parva*-infected cell lines and from IL-2-dependent lymphocyte controls were labelled *in vitro* in the protein kinase assays, boiled in SDS sample buffer for 5 min and then run on SDS–polyacrylamide (5–17.5% or 7.5–15%) gels according to Laemmli (1970). The gels were stained with Coomassie blue, destained and dried. When gels were to be treated with alkali, the dried gel was immersed into 10 vol of freshly prepared 1 M KOH and incubated in a covered container at 55°C for 2 h and then processed as described by Cooper *et al.* (1983). The dried gels were exposed to Fuji RX 100 film for autoradiography at −80°C with intensifying screens or to achieve a better resolution of phosphorylated protein bands, to Hyperfilm βmax (Amersham) at 23°C.

For analysis of phosphoamino acids, unstained protein bands were excised from preparative SDS–polyacrylamide gels, electroeluted, extensively

dialyzed against deionized water and lyophilized. Partial alkaline or acid hydrolysis was performed in 5 N KOH at 152°C for 35 min or in 6 N HCl at 110°C for 90 min, respectively (Martensen, 1984). The hydrolysates were analysed on a Model 324 Beckman HPLC equipped with an Ultrasil Ax anion exchange column (Altex Instruments Inc., Berkeley, CA). The column was equilibrated with 15 mM KH_2PO_4, pH 3.8 and the absorbance monitored at 210 nm. The sample was co-chromatographed with phosphoamino acid (Sigma) internal standards consisting of 200 μg phosphoserine, 150 μg phosphothreonine and 2–3 μg of phosphotyrosine. Upon sample injection a fraction collector was simultaneously activated and 0.25–0.5 ml fractions were collected at a rate of 1 ml/min. Pooled fractions from HPLC containing radioactivity or whole hydrolysates were run on thin layer electrophoresis to ascertain the identity of phosphoamino acids (Manai and Cozzone, 1982).

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References

Ackerman,P. and Osheroff,N. (1989) *J. Biol. Chem.*, **264**, 11958–11965.
Ackerman,P., Glover,C.V.C. and Osheroff,N. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 821–825.
Baldwin,C.L. *et al.* (1988) *Infect. Immun.*, **56**, 462–467.
Barbosa,M.S., Edmonds,C., Fisher,C., Schiller,J.T., Lowy,D.R. and Vousden,K.H. (1990) *EMBO J.*, **9**, 153–160.
Bensaid,A., Kaushal,A., Baldwin,C.L., Clevers,H., Young,J.R., Kemp,S.J., MacHugh,N.D., Toye,P.G. and Teale,A.J. (1991) *Immunogenetics*, **33**, 247–254.
Brown,C.G.D. (1983) In Jensen,J.B. (ed.), *In Vitro Cultivation of Protozoan Parasites*. CRC Press, Boca Raton, FL, pp. 243–284.
Brown,W.C. and Grab,D.J. (1985) *J. Immunol.*, **133**, 3184–3189.
Cardenas,M.E., Dang,Q., Glover,C.V.C. and Gasser,S.M. (1992) *EMBO J.*, **11**, 1785–1796.
Carroll,D. and Marshak,D.R. (1989) *J. Biol. Chem.*, **264**, 7345–7348.
Carroll,D., Santoro,N. and Marshak,D.R. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **LIII**, 91–95.
Chen-Wu,J.L.-P., Padmanabha,R. and Glover,C.V.C. (1988) *Mol. Cell. Biol.*, **8**, 4981–4990.
Cochet,C. and Chambaz,E.M. (1983) *J. Biol. Chem.*, **258**, 1403–1406.
Conrad,P.A., Iams,K.P., Brown,W.C., Sohanpal,B. and ole-MoiYoi,O.K. (1987) *Mol. Biochem. Parasitol.*, **25**, 213–226.
Cooper,G.M. (1990) *Oncogenes*, Jones and Bartlett Publishers, Boston, pp. 255–276.
Cooper,J.A., Sefton,B.M. and Hunter,T. (1983) *Methods Enzymol.*, **99**, 387–402.
Cross,M. and Dexter,T.M. (1991) *Cell*, **64**, 271–280.
Dahmus,G.K., Claiborne,C.V.C., Brutlag,D.L. and Dahmus,M.E. (1984) *J. Biol. Chem.*, **259**, 9001–9006.
Damuni,Z. and Reed,L. (1988) *Arch. Biochem. Biophys.*, **262**, 574–584.
DeBenedette,M. and Snow,E.C. (1991) *J. Immunol.*, **147**, 2839–2845.
Dobbelaere,D.A.E., Spooner.,P.R., Barry,W.C. and Irvin,A.D. (1984) *Parasite Immunol.*, **6**, 361–370.
Dobbelaere,D.A.E., Coquerelle,T.M., Roditi,M.E. and Williams,R.O. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4730–4734.
Dobrowolska,G., Meggio,F., Szczegielniak,J., Muszynska,G. and Pinna,L.A. (1992) *Eur. J. Biochem.*, **204**, 299–303.
Dozier,C., Denhez,F., Coll,J., Amouyel,P., Quatannes,B., Begue,A., Stehelin,D. and Saule,S. (1987) *Mol. Cell. Biol.*, **7**, 1995–1998.
Draetta,G. (1990) *Trends Biochem. Sci.*, **15**, 378–383.
Dyer,M., Hall,R., Shiels,B. and Tait,A. (1992) *Exp. Parasitol.*, **74**, 216–227.
Edelman,D.K., Blumenthal,D.K. and Krebs,E.G. (1987) *Annu. Rev. Biochem.*, **56**, 567–613.
Fawcett,D.W., Doxsey,S., Stagg,D.A. and Young,A.S. (1982) *Eur. J. Clin. Invest.*, **27**, 10–21.
Fell,A.H., Preston,P.M. and Ansell,J.D. (1990) *Parasite Immunol.*, **12**, 335–339.
Filhol,O., Cochet,C. and Chambaz,E.M. (1990) *Biochemistry*, **29**, 9929–9936.
Geahlen,R.L. and Harrison,M.L. (1984) *Biochim. Biophys. Acta*, **804**, 169–175.
Glineur,C., Bailly,M. and Ghysdael,J. (1989) *Oncogene*, **4**, 1247–1254.
Grasser,F.A., Scheidtmann,K.G., Tuazon,P.T. Traugh,J.A. and Walter,G. (1988) *Virology*, **165**, 13–22.
Hathaway,G.M. and Traugh,J.A. (1982) *Curr. Topics Cell. Regul.*, **21**, 101–127.
Hathaway,G.M. and Traugh,J.A. (1983) *Methods Enzymol.*, **99**, 308–331.
Hathaway,G.M. and Traugh,J.A. (1984) *J. Biol. Chem.*, **259**, 7011–7015.
Heldin,C.-H. and Westermark,B. (1984) *Cell*, **37**, 9–20.
Heldin,C.-H. and Westermark,B. (1990) *J. Cell Sci.*, **96**, 193–196.
Heller-Harrison,R.A. and Czech,M.P. (1991) *J. Biol. Chem.*, **266**, 14435–14439.
Heller-Harrison,R.A., Meisner,H. and Czech,M.P. (1989) *Biochemistry*, **28**, 9053–9058.
Hu,E. and Rubin,C.S. (1990) *J. Biol. Chem.*, **263**, 5072–5080.
Iams,K.P., Young,J.R., Nene,V., Desai,J., Webster,P., ole-MoiYoi,O.K. and Musoke,A.J. (1990) *Mol. Biochem. Parasitol.*, **39**, 47–60.
Irvin,A.D., Brown,C.G.D., Kanhai,G.K. and Stagg,D.A. (1975) *Nature*, **255**, 713–714.
Ivanov,V., Stein,B., Baumann,I., Dobbelaere,D.A.E., Herrlich,P. and Williams,R.O. (1989) *Mol. Cell. Biol.*, **9**, 4677–4686.
Klarlund,J.K. and Czech,M.P. (1988) *J. Biol. Chem.*, **263**, 15872–15875.
Krebs,E.G., Eisenman,R.N., Kuenzel,E.A., Litchfield,D.W., Lozeman,F.J. and Lüscher,B. and Sommercorn,J. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 77–84.
Krek,W., Maridor,G. and Nigg,E.A. (1992) *J. Cell Biol.*, **116**, 43–55.
Laemmli,U.K. (1970) *Nature*, **277**, 680–685.
Lin,W.-J., Tuazon,P.T. and Traugh,J.A. (1991) *J. Biol. Chem.*, **266**, 5664–5669.
Litchfield,D.W., Lozeman,F.J., Piening,C., Sommercorn,J., Takio,K., Walsh,K.A. and Krebs,E.G. (1990) *J. Biol. Chem.*, **265**, 7638–7644.
Lozeman,F.J., Litchfield,D.W., Piening,C., Takio,K., Walsh,K.A. and Krebs,E.G. (1990) *Biochemistry*, **29**, 8436–8447.
Lüscher,B., Kuenzel,E.A., Krebs,E.G. and Eisenman,R.N. (1989) *EMBO J.*, **8**, 1111–1119.
Lüscher,B., Christianson,E., Litchfield,D.W., Krebs,E.G. and Eisenman,R.N. (1990) *Nature*, **344**, 517–522.
Manai,M. and Cozzone,A.J. (1982) *Anal. Biochem.*, **124**, 12–18.
Maridor,G., Park,W., Krek,W. and Nigg,E.A. (1991) *J. Biol. Chem.*, **266**, 2362–2368.
Martensen,T.M. (1984) *Methods Enzymol.*, **107**, 3–23.
Meek,D.W., Simon,S., Kikkawa,U. and Eckhart,W. (1990) *EMBO J.*, **9**, 3253–3260.
Meisner,H. and Czech,M.P. (1991) *Curr. Opin. Cell Biol.*, **3**, 474–483.
Meisner,H., Heller-Harrison,R., Buxton,J. and Czech,M.P. (1989) *Biochemistry*, **28**, 4072–4076.
Münstermann,U., Fritz,G., Seitz,G., Yiping,L., Schneider,H.R. and Issinger,O.-G. (1990) *Eur. J. Biochem.*, **189**, 251–257.
Morrison,D.K., Kaplan,D.R., Escobedo,J.A., Rapp,U.R., Roberts,T.M. and Williams,L.T. (1989) *Cell*, **58**, 649–657.
Morrison,W.I., Goddeeris,B.M. and Teale,A.J. (1987) *Eur. J. Immunol.*, **17**, 1703–1709.
Mullner-Lorillon,O., Marot,J., Cayla,X., Pouhle,R. and Bellé,R. (1988) *Eur. J. Biochem.*, **171**, 107–117.
Mullner-Lorillon,O., Cormier,P., Labbé,J., Doreé,M., Pouhle,R., Osborne,H. and Bell (1990) *Eur. J. Biochem.*, **193**, 529–534.
Nigro,J.M., Sikorski,R., Reed,S.I. and Vogelstein,B. (1992) *Mol. Cell. Biol.*, **12**, 1357–1365.
ole-MoiYoi,O.K., Sugimoto,C., Conrad,P.A. and Macklin,M.D. (1992) *Biochemistry*, **31**, 6193–6202.
Padmanabha,R., Chen-Wu,J.L.-P., Hanna,D.E. and Glover,C.V.C. (1990) *Mol. Cell. Biol.*, **8**, 4089–4099.
Pinna,L.A. (1990) *Biochim. Biophys. Acta*, **1054**, 267–284.
Rihs,H.-P., Jans,D.A., Fan,H. and Peters,R. (1991) *EMBO J.*, **10**, 633–639.
Sanghera,J.S., Charlton,L.A., Paddon,H.B. and Pelech,S.L. (1992) *Biochem. J.*, **283**, 829–837.
Saxena,A., Padmanabha,R. and Glover,C.V.C. (1987) *Mol. Cell. Biol.*, **7**, 3409–3417.
Siegel,J.N., Egerton,M., Phillips,A.F. and Samuelson,L.E. (1991) *Semin. Immunol.*, **3**, 325–334.
Singh,T.J. and Huang,K. (1985) *FEBS Lett.*, **190**, 84–88.

Sommercorn,J. and Krebs,E.G. (1987) *J. Biol. Chem.*, **262**, 3839–3843.
Sommercorn,J., Mulligan,J.A., Lozeman,F.J. and Krebs,E.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 38–8834.
Takio,K., Kuenzel,E.A., Walsh,K.A. and Krebs,E.G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4851–4855.
Taylor,A., Allende,C.A., Weinman,R. and Allende,J.E. (1987) *FEBS Lett.*, **226**, 109–114.
Tuazon,P.T. and Traugh,J.A. (1991) *Adv. Second Messenger Phosphoprotein Res.*, **23**, 123–164.
Yang-Feng,T.L., Zeng,K., Kopatz,I., Naiman,T. and Canaani,D. (1991) *Nucleic Acids Res.*, **19**, 7125–7129.
Yu,I.J., Spector,D.L., Bae,Y.-S. and Marshark,D.R. (1991) *J. Cell Biol.*, **114**, 1217–1232.

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