Protein kinases in divergent eukaryotes: Identification of protein kinase activities regulated during trypanosome development

(Giardia lamblia/Trichomonas vaginalis/tyrosine phosphorylation)

Marilyn Parsons*, Mary Valentine§, and Victoria Carter*

*Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, WA 98109; and Departments of Pathobiology and Microbiology, University of Washington, Seattle, WA 98195

Communicated by George J. Todaro, September 8, 1992

ABSTRACT The role of protein kinases in organisms that diverged early in the eukaryotic lineage is relatively unexplored. In this study, we determined that primitive parasitic protozoa possess multiple protein-serine kinases and inferred the presence of protein-tyrosine kinases through sensitive immunoblotting techniques. To further explore the role of protein kinases in parasite development, we examined the activity of eight renaturable protein kinases during the life cycle of the protozoan parasite Trypanosoma brucei. The activities of six protein-serine/threonine kinases were regulated during development, with several distinct patterns of regulation. In addition, an 89-kDa protein kinase was detected in dividing cells but not in nondividing cells. Our data indicate that even the most primitive eukaryotes possess a large complement of protein kinases, including protein-tyrosine kinases as well as protein-serine/threonine kinases. The data further suggest that protein kinases may play a pivotal role in regulation of proliferation and differentiation in protozoa.

In higher eukaryotes, regulation of the complex interactions required for differentiation and proliferation is mediated in part by protein phosphorylation networks. The role of protein phosphorylation in organisms that diverged early in the eukaryotic lineage has not yet been studied in detail. The extant representatives of these most divergent lines are certain protozoan parasites, including those studied here: Trypanosoma brucei, Trichomonas vaginalis, and Giardia lamblia. Analysis of rRNA phylogenies indicates that T. brucei and G. lamblia are, respectively, 2 and 3 times more distant from mammals than is yeast, with T. vaginalis showing intermediate divergence (1). The ancient origins of these organisms are reflected in their unusual cytological organization [e.g., the lack of mitochondria in T. vaginalis and G. lamblia (2)] and management of genetic information [e.g., RNA editing and obligate trans-splicing in T. brucei (3–5)]. The goals of this study were to determine whether these most ancient eukaryotes possess a full complement of protein kinases and whether these protein kinases might play a role in their development.

T. brucei undergoes episodes of proliferation and differentiation and has several developmental stages (6). Differentiation is cyclic rather than terminal. In the mammalian host, dividing slender blood forms differentiate to the insect-infective, nondividing intermediate and stumpy blood forms. Forms analogous to procyclic forms, which reside in the tsetse fly midgut, can be cultured in vitro. Many functions, including glycolysis (7), mitochondrial respiration (8), RNA editing (3), endocytosis (9), as well as infectivity (10) and membrane composition (9, 11–13) are stage regulated. By analogy with higher organisms, such global changes could be controlled, in part, by protein phosphorylation.

If protein phosphorylation plays an important role in parasite differentiation, the activity of many protein kinases and phosphatases might be regulated during the life cycle. Only a few protein kinase activities have been studied for their stage regulation in T. brucei. Protein kinase C-like activities are apparently restricted to blood forms (14) and casein kinase-like activity is 3-fold higher in slender than in stumpy blood forms (15). Recently, we have shown that T. brucei contains a number of stage-regulated tyrosine-phosphorylated species, suggesting potential stage regulation of tyrosine phosphorylation networks (16).

The present study directly examines the activity of individual protein kinases in various developmental stages of T. brucei. Since there was so little information about protein kinases in these organisms, we did not wish to restrict our analyses to those kinases already described. Therefore, we used a renaturation assay that allows analysis of a range of individual protein kinases. Our data indicate that even the most divergent eukaryotes probably possess sophisticated protein phosphorylation networks.

MATERIALS AND METHODS

Parasites. Blood samples and midlogarithmic-phase procyclic forms of the pleomorphic (differentiation competent) strain TREU667 and monomorphic strain EATRO164 clone IHR11 were provided by C. C. Wang and Alice Wang (University of California at San Francisco), and T. vaginalis trichomonads were kindly provided by Don Riley (University of Washington). Washed cells were lysed in SDS/PAGE sample buffer containing 2-mercaptoethanol, boiled, and frozen at -70°C until use.

Kinase Renaturation Assay and Phosphoamino Acid Analysis. SDS/10% polyacrylamide gels were run until the 30-kDa prestained marker (BRL) neared the bottom of the gel and the proteins were transferred to poly(vinylidene difluoride) membranes (17). The renaturation assay followed the procedure of Ferrell and Martin (17), except that the labeling in 30 mM Tris-HCl or Hepes-KOH, pH 7.5/2 mM MnCl2/10 mM MgCl2/50 μCi of [γ-32P]ATP per ml (1 Ci = 37 GBq) (Gammaprep: Promega) was extended to 1 hr (similar results were obtained in both buffers). Quantitation of radioactivity associated with specific bands was performed with a Molecular Dynamics phosphorimager and ImageQuant software (18). Direct comparisons were made only between samples on the same blot. In some experiments, radiolabeled bands were excised, hydrolyzed in 5.7 M HCl for 1 hr at 110°C, and subjected to two-dimensional phosphoamino acid analysis with a CBS Scientific (Del Mar, CA) apparatus (19).

Phosphorylation of Exogenous Substrates. Three lanes containing 50 μg of protein were used for each sample. After renaturation in buffer without bovine serum albumin, the
three lanes were cut into 0.5-cm strips and incubated in 100 μl of buffer containing 30 mM Tris-HCl, 10 mM MgCl₂, 2 mM MnCl₂, 200 μCi of [γ-³²P]ATP per ml, and 0.1 mg each of histone H1, enolase, dephosphorylated β-casein, and myelin basic protein per ml. After 20 min at room temperature, the reactions were terminated by addition of 5× SDS sample buffer. One-half of the reaction products were separated by SDS/PAGE on an 11% polyacrylamide gel and revealed by autoradiography.

Anti-phosphotyrosine Western Blot Analysis. Affinity-purified anti-phosphotyrosine antibodies were a gift of Jeffrey Ledbetter and Gary Schieven (Pharmaceutical Research Institute of Bristol-Myers Squibb) and are the same antibodies used in our previous work on T. brucei (16). The procedures are also the same.

RESULTS

Multiple Renaturable Protein Kinases in T. brucei. The kinase renaturation assay allows detection of individual protein kinases present in mixtures of cellular proteins. Proteins are separated by SDS/PAGE, transferred to poly(vinylidene difluoride), and then renatured. After incubation with [γ-³²P]ATP, radiolabeled bands are revealed by autoradiography, indicating the location of that subset of protein kinases that regain activity. Most of the phosphorylation observed is autophosphorylation, but phosphorylation of comigrating substrates and/or the blocking agent could also occur (17). Since different protein kinases do not renature equally well, and since all are assayed under the same conditions regardless of their own optima, it is not possible to compare activities of different protein kinases with this assay.

As shown in Fig. 1A, this assay revealed multiple bands in T. brucei procyclic cell lysates, suggesting the presence of protein kinases ranging in size from 43 to 123 kDa. Very low concentrations of nonradioactive ATP blocked the labeling, while GTP competed only at 100-fold higher concentrations. Varying the pH and salt concentration revealed no additional kinases and indicated that the renaturable kinases were most active at pH values ranging from 6.5 to 7.5 and at 0–100 mM KCl (data not shown). Protein kinases require the presence of metal ions for activity. As shown in Fig. 1B, no radiolabeled bands were observed in the absence of metal ions. Bands were maximally labeled in the presence of manganese alone and to a lesser degree when only magnesium was present.

To rule out the possibility that the radiolabeled bands represent unusual ATP binding proteins and to determine the substrate specificity of the protein kinases, phosphoamino acid analysis was performed on individual bands. Summarized in Table 1 are the results for all protein kinases examined in this study, including those more abundant in other stages of the T. brucei life cycle (see Fig. 2A) and those identified in other protozoan species (see Fig. 4A). All of the bands contained radiolabeled phosphoserine and/or phosphothreonine, indicating the covalent attachment of phosphate to these residues. Thus, the bands reveal the sites of active protein kinases on the blot. Trace amounts of phosphotyrosine were consistently detected in the PK97 band. These data are similar to what was found in other renaturation studies, in which phosphotyrosine is rarely observed (17).

Developmental Regulation. Phosphorimaging directly quantitates radioactivity on the blot; its linear dynamic range of 100,000 allows comparison of widely varying activities with a single exposure. In control experiments, analysis of a dilution series of cell extracts indicated that the relationship between protein concentration and ³²P incorporation into specific bands as revealed by phosphorimaging was linear from 6 to at least 40 μg. To determine whether the activities of individual protein kinases were developmentally regulated, samples from various stages of the T. brucei life cycle were examined. A representative analysis of the naturally differentiating strain TREU667 is shown in Fig. 2A. The activity of most of the protein kinases varied during development. Numerous blots and independently prepared samples were examined with similar results. To derive quantitative data, three independent samples from each stage were analyzed by the renaturation assay followed by phosphorimaging. Shown in Fig. 2B is the quantitation obtained for those protein kinases that were clearly identifiable on multiple blots. With the exception of PK97, all activities varied between stages. Several distinct patterns were observed, with specific protein kinases showing highest activities in slender (PK181, PK123, PK43), or procyclic forms (PK52). The activities of the kinases varied somewhat within stages, probably reflecting in part our sampling along

<table>
<thead>
<tr>
<th>T. brucei</th>
<th>T. vaginalis</th>
<th>G. lamblia</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td>PS</td>
<td>PT</td>
</tr>
<tr>
<td>181</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>123</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>97</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>69</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>75</td>
<td>+++</td>
<td>±</td>
</tr>
<tr>
<td>71</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>64*</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>52</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>47</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td>+++</td>
<td>–</td>
</tr>
</tbody>
</table>

PS, phosphoserine; PT, phosphothreonine. The most abundant phosphoamino acid in a sample was arbitrarily assigned a value of ++; other phosphoamino acids were assigned values as follows: ++, similar abundance; +, about half as abundant; ±, substantially less abundant; –, visible only upon overexposure; –, not visible. No phosphotyrosine was observed, except for low levels (±) in band PK97.

*Upper band of doublet was analyzed.

Fig. 1. Kinase renaturation assay reveals multiple species in T. brucei procyclic forms. Bands referred to in the text are identified by molecular mass (kDa) (PK64D is a doublet). Between the blots, migration of marker proteins is indicated. Fifty micrograms of protein was loaded per lane. (A) Analysis of nucleotide specificity. Included in the labeling reaction mixture were the nonradioactive nucleotides at the indicated micromolar concentrations. Mn + Mg, 2 mM MnCl₂ and 10 mM MgCl₂. Reactions were performed in Hepes/KOH buffer.
forms. The protein kinases identified here appear to be distinct from those characterized by others (14, 20), as they differ in molecular mass, substrate specificity, and/or pattern of stage regulation.

Unlike slender blood forms and procyclic forms, stumpy blood forms are nondividing cells. To determine whether the changes in protein kinase activity observed in stumpy forms were the result of cell cycle regulation, stationary-phase procyclic forms were prepared (Fig. 2A, lanes PF-S). The protein kinase activities induced in stumpy forms were not induced in these cells. However, PK89 activity was almost absent in stationary procyclic forms. Thus, PK89 activity is depressed in nonproliferating cells of both blood form and procyclic stages, suggesting a potential role for this molecule in cell cycle progression. The activity of other protein kinases often decreased in stationary phase, but these results were not as reproducible or dramatic.

It is probable that the stage-regulated activities observed do not reflect nonspecific interference with comigrating proteins since the bulk of these proteins are not stage regulated (21). The alterations in activity observed could be due to changes in kinase abundance or specific activity or to changes in substrate abundance or phosphorylatability (either the kinase itself or a comigrating substrate). To address the latter possibilities, we assayed the protein kinase activities in the presence of exogenous potential substrates: histone H1, enolase, myelin basic protein, and casein. After blotting and renaturation, lanes were cut into strips, which were incubated in labeling buffer containing exogenous substrates and \([\gamma-^3P]ATP\). The positions of the radiolabeled bands following electrophoretic separation indicated which of the potential substrates were phosphorylated. Myelin basic protein proved to be a useful substrate for the protein kinases shown in Fig. 3. (The other substrates were not efficiently phosphorylated by any of the protein kinases; data not shown.) In each case, the stage regulation of \(^3P\) incorporation into myelin basic protein paralleled its incorporation into the kinase region on the blot. Thus, the stage regulation observed reflects changes in the protein kinase abundance or specific activity and not alterations in substrates. However, since the renaturation assay examines protein kinases outside their normal cellular milieu, the regulation we observed may not in every case parallel that which occurs within the cell.

the continuum of the differentiation process. PK47 levels in slender forms were most variable, ranging from the low levels seen in procyclic forms to the high levels seen in stumpy

FIG. 2. Stage regulation of renaturable protein kinases. (A) Renaturation analysis. Cell lysates were prepared from various stages of the differentiation-competent strain TREU667 and the nondifferentiating strain EATRO164. SL, slender blood forms; ST, stumpy blood forms; D-ST, difluoromethylornithine-induced stumpy blood forms; PF, procyclic forms; PF-S, stationary procyclic forms. (Left) Bands referred to in the text are identified by molecular mass (kDa). (Right) Migration of marker proteins is indicated. All lanes are from the same autoradiographic exposure of one gel. Approximately 50 \(\mu g\) of protein was loaded per lane. (B) Quantitation of developmental regulation. Three independent samples of each developmental stage of \(T. \) brucei TREU667 were analyzed on one blot (25 \(\mu g\) per lane) and radioactivity associated with each band was determined by phosphorimaging. Values for \(^3P\) incorporation are given in arbitrary units obtained from one exposure.

FIG. 3. Developmental regulation of phosphorylation of myelin basic protein by renatured kinases. As described, the ability of particular renatured kinases to phosphorylate myelin basic protein was measured by incubating horizontal strips of blots with exogenous substrates. SL, slender blood forms; ST, stumpy blood forms; PF, procyclic forms. For each sample, both autophosphorylation (Auto) and phosphorylation of myelin basic protein (MBP) by specific regions of the gel are shown. For autophosphorylation, 50 \(\mu g\) of total cell protein was loaded. MBP panels show phosphorylation by strips from lanes containing 75 \(\mu g\) of cell protein. Bands are identified by molecular mass (kDa).
We also examined the activities of renaturable protein kinases in *T. brucei* EATRO164, which is defective in the slender to stumpy transition (Fig. 2A). Slender blood forms of this strain showed substantially lower PK181 and PK123 activity than the differentiation-competent strain. When forced to transform to morphologically stumpy forms by treatment with difluoromethylornithine, the activities of several protein kinases were decreased. PK123 and PK181 activities were induced but remain far below the levels found in naturally differentiating slender and stumpy forms. Since difluoromethylornithine-induced stumpy forms show abnormal regulation of protein kinase activities and decreased tyrosine phosphorylation (16), it appears that drug treatment may not induce normal developmental changes in protein phosphorylation networks.

**Renaturable Kinases and Tyrosine Phosphorylation in Ancient Eukaryotes.** Parasites related to *T. brucei* and even more ancient parasites possess multiple protein kinases that can be revealed in renaturation studies. Fig. 4A shows the renaturable kinases observed in various cells ranging from human carcinoma cells to *Escherichia coli*. Kinoplastid parasites related to *T. brucei* such as *Crithidia fasciculata*, as well as *Trypanosoma cruzi* epimastigotes and *Leishmania chagasi* promastigotes (data not shown) possess multiple renaturable protein kinases, as do the unrelated protozoa *T. vaginalis* and *G. lamblia*. Phosphoamino acid analyses of selected bands from these most divergent eukaryotes showed the presence of phosphoserine and phosphothreonine (Table 1). In contrast, kinase renaturation studies of *E. coli* lysates showed few if any bands (the smear on the lane is from adjacent *G. lamblia* samples).

We wished to determine whether these parasites possessed a full complement of protein kinases, including protein-tyrosine kinases. Since the renaturation assay reveals primarily serine/threonine kinases and since protein-tyrosine kinases are generally low-abundance proteins, we turned to a sensitive immunoblotting technique that detects tyrosine-phosphorylated proteins. Affinity-purified anti-phosphotyrosine antibodies were used in Western blot analysis. Fig. 4B shows that unlike *E. coli* all eukaryotic species tested possessed multiple proteins that react with the anti-phosphotyrosine antibodies. *G. lamblia* and *T. vaginalis* are particularly rich in such proteins, since the autoradiographic exposure is only one-fourth that of the other species. The binding of the antibodies to the parasite proteins was completely blocked by the relevant hapten phenyl phosphate, demonstrating the specificity of the reaction (data not shown). These data provide evidence for the existence of protein-tyrosine kinases in these organisms.

**DISCUSSION**

Evolution of the eukaryotic cell, including the nucleus, endomembrane system, and cytoskeleton, must have required an increased ability to integrate regulatory functions. Higher eukaryotes integrate these functions in part through complex phosphorylation networks, which are modulated by protein kinases and phosphatases. Did these networks evolve with the earliest eukaryotes? To explore this question we studied one component of the networks: protein kinases. We found that the extant representatives of the most ancient lineages possess abundant renaturable protein-serine/threonine kinases. Evidence for protein-tyrosine kinases in primitive parasitic protozoa was obtained through the detection of tyrosine-phosphorylated proteins. Together these data suggest that evolution of the eukaryote was accompanied by a major increase in complexity of protein phosphorylation networks.

In higher eukaryotes, one role of phosphorylation networks is to regulate pathways required for cell proliferation and differentiation (22–27). If protein kinases are involved in the cyclic differentiation of *T. brucei*, we reasoned that the activities of a subpopulation of protein kinases would also be cyclically regulated. Still, it was surprising to us that the activities of six of eight renaturable protein kinases were regulated during the life cycle and that the seventh was apparently regulated during the cell cycle. The elevated activity of several protein kinases in stumpy forms is intriguing since these cells are thought to be situated at a major regulatory point within the life cycle. In *Leishmania major*, the infective, stationary-phase metacyclic forms (another transitional developmental stage) contain much higher his-
tone kinase activity than noninfective, logarithmic-phase cells (28). Based on the findings described above, as well as our previous results demonstrating stage regulation of tyrosine-phosphorylation patterns (16), we hypothesize that protein phosphorylation networks are important in regulating the cyclical development of *T. brucei*. In support of this hypothesis, we found that a strain defective for the slender to stumpy transformation had decreased activity of both PK181 and PK123.

In addition to potential roles in regulation of parasite functions, it is also possible that parasite protein kinases modulate host functions. For example, *Leishmania* possesses an extracellular protein kinase with the ability to phosphorylate complement components (29). The roles of specific protein kinases can be fully understood only through manipulation of the parasite genome. In this paper, we have identified several protein kinases that would be suitable targets for genetic studies once the relevant genes are cloned.

The authors thank Drs. Jeffrey Ledbetter, Gary Schieven, and Randall Howard for helpful discussions. We also thank Dr. Laurie Read and Dr. Jeffrey Ledbetter for critical evaluation of the manuscript and Karen Licciardi for technical assistance. This work was supported in part by National Institutes of Health Grants AI22635, AI31077, and GM42508 and by The Murdock Charitable Trust.