Volume 222, number 1, 104–108 FEB 05089 September 1987

Demonstration that LSTRA cells have an elevated level of proteins phosphorylated on tyrosine residues

Rebecca E. Thom and John E. Casnellie

Department of Pharmacology and Cancer Center, The University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

Received 4 July 1987

The LSTRA cell line has been shown to have an exceptionally high level of a tyrosine protein kinase ($pp56^{lck}$). We now report that LSTRA cells also have a much higher level of proteins phosphorylated on tyrosine residues in comparison to several other cell lines with normal levels of $pp56^{lck}$. The level of phosphotyrosine-containing proteins in LSTRA cells was comparable to that seen in K562 cells, a cell line known to have a constitutively active tyrosine protein kinase. These results provide evidence that LSTRA cells have an elevated level of in vivo tyrosine protein kinase activity, probably due to the overexpression and activation of $pp56^{lck}$.

Tyrosine protein kinase; Phosphotyrosine antibody; (Lymphoid cell)

1. INTRODUCTION

The LSTRA cell line contains an elevated level of a tyrosine protein kinase ($pp56^{lck}$) with especially strong sequence homology with $pp60^{src}$ [1–4]. It has been suggested that the elevated level of $pp56^{lck}$ could have a causal role in the transformation of LSTRA cells [2,4]. In analogy with other tyrosine protein kinases such as $pp60^{src}$ [5,6], a transforming role for $pp56^{lck}$ requires that its overexpression results in an increase in the in vivo level of tyrosine protein kinase activity in these cells. Although it has been conclusively demonstrated that $pp56^{lck}$ is elevated in LSTRA cells, it is not known if this results in an increase in the tyrosine phosphorylation of proteins other than $pp56^{lck}$. This is an important point because overexpression of $pp60^{src}$ fails to result in an increased level of in vivo tyrosine protein kinase activity since the enzyme is in an inactive form [5,6].

We have shown that in LSTRA cells $pp56^{lck}$ is phosphorylated in vivo at several sites on tyrosine residues [7], one of them being the site of in vitro autophosphorylation. This site is identical in sequence to the site of autophosphorylation in $pp60^{src}$ [7]. In $pp60^{src}$ this site is phosphorylated only when $pp60^{src}$ is in an active form [8]. Given the strong sequence homology between $pp56^{lck}$ and $pp60^{src}$ [3,4], it seems likely that in $pp56^{lck}$ this site is also only phosphorylated in catalytically active molecules, and therefore LSTRA cells should have a high intracellular level of tyrosine protein kinase activity. We have previously shown that LSTRA cells have a relatively high phosphotyrosine content [1], and this was interpreted as indicating an enhanced level of tyrosine protein kinase activity. However, these results could simply reflect the elevated level of $pp56^{lck}$ in these cells since this protein is highly phosphorylated on tyrosine residues [7]. A more definitive approach to measuring in

Correspondence address: J.E. Casnellie, Department of Pharmacology and Cancer Center, The University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, USA

Abbreviations: $pp56^{lck}$, tyrosine protein kinase of M_r 56000 found elevated in LSTRA cells; $pp60^{src}$, the cellular homologue of the transforming protein of the Rous sarcoma virus; $p210^{c\text{-}abl}$, form of the Abelson tyrosine protein kinase expressed in K562 cells

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50 © 1987 Federation of European Biochemical Societies

vivo levels of tyrosine protein kinase activity is through the use of antibodies to phosphotyrosine. These antibodies permit one to monitor the general level of phosphotyrosine-containing proteins in intact cells and thus obtain a measure of the level of in vivo tyrosine protein kinase activity.

2. EXPERIMENTAL

Unless otherwise indicated the antibodies to phosphotyrosine were prepared using phosphotyrosine conjugated to human immunoglobulin as the antigen [9,10]. Rabbits were initially immunized by injection into the popliteal lymph nodes followed by intradermal booster injections. The antibodies were affinity purified on a phosphotyrosine-Sephadex column using 25 mM phosphotyrosine to elute the specific antibodies. Antibodies were first dialyzed against 3 changes of 1 l volumes of phosphate-buffered saline to remove the phosphotyrosine and then concentrated to about 10 mg/ml by dialysis against concentrated sucrose. Some experiments were performed with phosphotyrosine antibodies prepared against the phosphotyrosine-containing Abelson protein expressed in bacteria [11], kindly provided by Dr J.Y.J. Wang (University of California at San Diego). Samples were subjected to electrophoresis on 7.5% polyacrylamide gels followed by transfer to nitrocellulose. Immunoblots were performed as previously described with ^{125}I-labeled protein A being used to detect the immune complexes [10]. Cells were cultured in Iscove's medium supplemented with 10% Nuserum (Collaborative Research). Primary cultures of interleukin-2-dependent T cells were established from PHA stimulated blasts as described in [10].

3. RESULTS

Initial experiments were aimed at characterizing the phosphotyrosine-containing proteins in LSTRA cells and demonstrating the specificity of the antibodies. In fig.1 intact LSTRA cells were dissolved in SDS and subjected to polyacrylamide electrophoresis followed by blotting to nitrocellulose. The blot in fig.1A was probed with antibodies prepared against phosphotyrosine conjugated to human immunoglobulin. The major

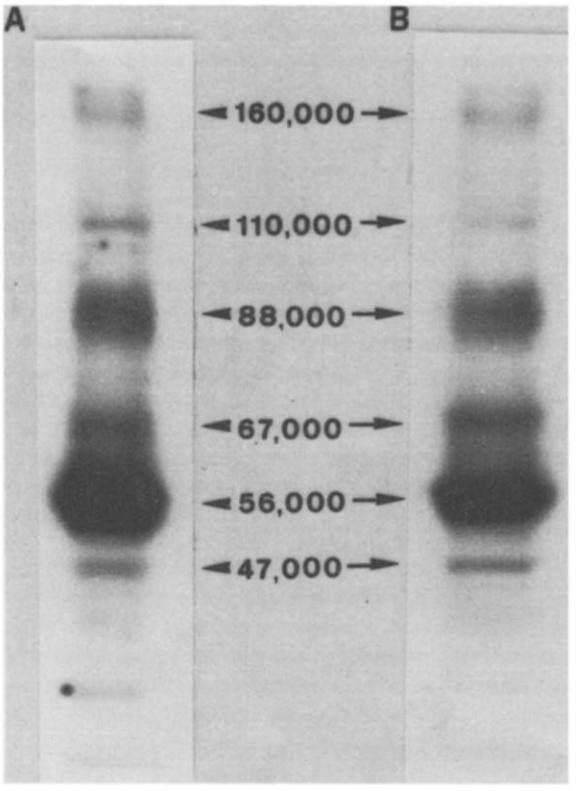

Fig.1. Pattern of proteins recognized in blots of LSTRA cells by antiphosphotyrosine antibodies made against phosphotyrosine conjugated to human immunoglobulin (A) or made against the phosphotyrosine-containing Abelson tyrosine protein kinase produced in bacteria (B). For each lane 1×10^6 cells were applied to the gel that was subsequently blotted onto nitrocellulose.

protein recognized by these antibodies has an M_r of 56000. We have previously identified this protein as pp56^{lck} [10]. In addition to pp56^{lck}, the antibodies recognize several other proteins in LSTRA cells, the most prominent ones having approximate molecular masses of 160000, 110000, 88000, 67000 and 47000.

In fig.1B the blot of LSTRA cells was probed with a preparation of phosphotyrosine antibodies made against the Abelson tyrosine protein kinase produced in bacteria [11]. The pattern of proteins recognized by these second antibodies was identical to that obtained with the antibodies made against the phosphotyrosine-immunoglobulin conjugates. Since the two antibodies were made using different carrier proteins, the only common

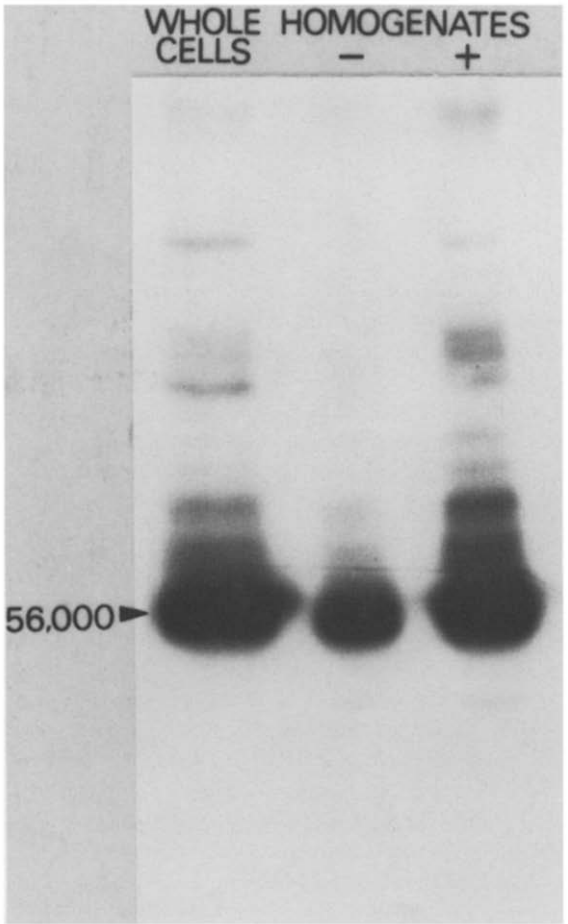

Fig.2. Preservation by phosphatase inhibitors of proteins recognized in homogenates of LSTRA cells by antibodies to phosphotyrosine. Cells were either immediately dissolved in electrophoresis sample buffer (WHOLE CELLS) or homogenized in 5 mM Hepes, pH 7.4, 1 mM EDTA, and incubated for 2 h on ice in the absence (–) or presence (+) of 1 mM sodium orthovanadate and 5 mM *p*-nitrophenyl phosphate. Material from an equivalent number of cells (1×10^6) was loaded onto each lane.

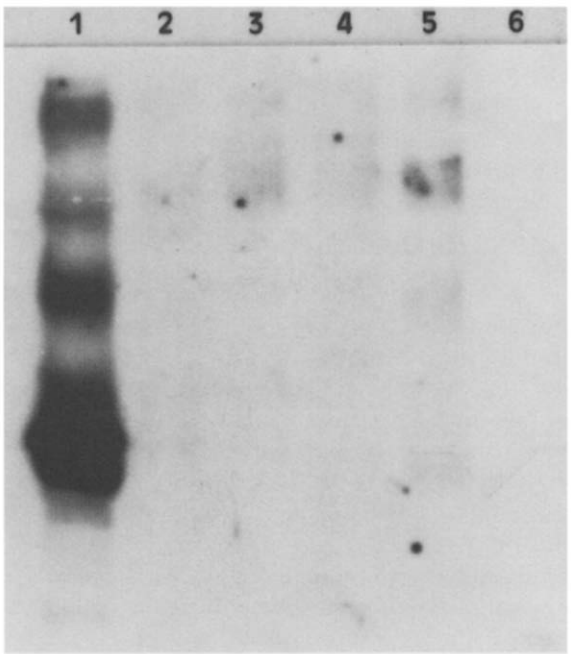

Fig.3. Autoradiogram of blot of various T cell lines probed with antibodies against phosphotyrosine. (1) LSTRA cells; (2) Jurkat cells; (3) CCRF-CEM cells; (4) 2B4 cells; (5) YAC cells; (6) interleukin-2-dependent T cells. The arrow indicates $pp56^{lck}$.

epitope that they should recognize is the phosphotyrosine moiety. Thus, the observation that two different antibodies recognize the same pattern of proteins provides strong evidence that they are recognizing phosphotyrosine residues in these proteins and not some cross-reacting but irrelevant epitope.

Additional control experiments confirmed this conclusion. Preincubation of the antibodies with phosphotyrosine completely inhibited their ability to detect proteins in LSTRA cells (not shown). Fig.2 shows that the proteins detected in the blots have another property expected of phosphotyrosine-containing proteins. The phosphotyrosine in these proteins should be vulnerable to phosphatases and undergo dephosphorylation in homogenates, resulting in the loss of the proteins from the blots. Such dephosphorylation should be inhibited by phosphotyrosine phosphatase inhibitors. Fig.2 shows that incubation of homogenates of LSTRA cells for 2 h on ice results in the loss of most of the proteins recognized by the phosphotyrosine antibodies. This loss is completely inhibited by the inclusion of the phosphotyrosine phosphatase inhibitors [12] sodium orthovanadate and *p*-nitrophenol phosphate. Thus the results from these various experiments provide conclusive evidence that the proteins identified in the blots are phosphorylated on tyrosine residues.

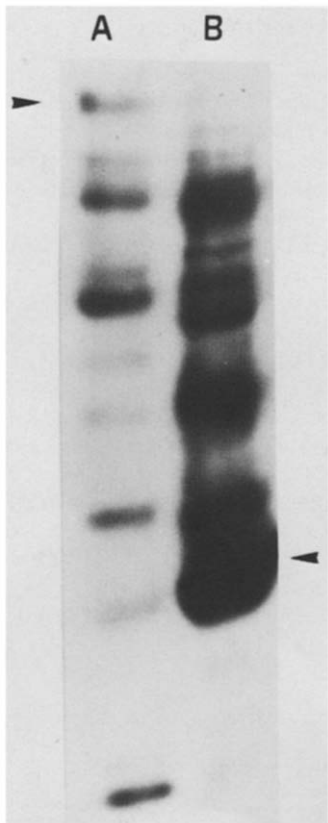

Fig.4. Comparison of the levels of proteins phosphorylated on tyrosine residues in K562 (A) and LSTRA cells (B). The blots were performed using 1×10^6 cells. This represented approximately twice as much protein in the sample of K562 cells as compared to the LSTRA cells. The arrowheads in A and B point to the positions of $p210^{c\text{-}abl}$ and $pp56^{lck}$, respectively.

Fig.3 compares the level of phosphotyrosine containing proteins in LSTRA cells (lane 1) with that in several other T cell lines (lanes 2–5), plus normal interleukin-2-dependent T cells (lane 6). For this experiment 2×10^6 cells were solubilized and loaded onto the gels. This represented approximately equal amounts of protein for each cell type. In contrast to the blots of LSTRA cells, these various other cells have very low levels of phosphotyrosine containing proteins. The result in fig.3 was obtained with the antibodies made against the phosphotyrosine-immunoglobulin conjugate. Similar results were also obtained with the antibodies made against the Abelson protein (not shown). The observation that the phosphotyrosine antibodies detected little or no $p56^{lck}$ in cells other than LSTRA is in agreement with results obtained with antibodies against a synthetic fragment of $p56^{lck}$, which showed that these cells have much lower levels of $pp56^{lck}$ in comparison to the LSTRA cell line (not shown).

In order to compare the level of phosphotyrosine-containing proteins in LSTRA cells with that of a cell line known to have a constitutively active tyrosine protein kinase, a blot was performed comparing LSTRA cells with K562 cells. The K562 cells are a nonadherent, hematopoietic line that have an elevated level of tyrosine protein kinase activity due to the expression of an aberrant, catalytically active form of the Abelson tyrosine protein kinase [13]. Previous workers have shown that this high level of in vivo tyrosine protein kinase activity causes the K562 cells to have a much higher level of phosphotyrosine-containing proteins in comparison to other cell lines [14]. As shown in fig.4, while the two cell lines have only a few phosphotyrosine-containing proteins in common, it is apparent that the overall level of phosphotyrosine-containing proteins in LSTRA cells is comparable to, if not greater than the level in K562 cells.

4. DISCUSSION

This work demonstrates that the LSTRA cell line has a much higher level of proteins phosphorylated on tyrosine residues than other T cell lines. The level of proteins phosphorylated on tyrosine residues in LSTRA cells was comparable to that seen in a cell line that is known to have a constitutively active tyrosine protein kinase. Thus, the regulation of protein tyrosine phosphorylation in the LSTRA cells is clearly abnormal. It is possible that these proteins are all constitutively phosphorylated on tyrosine residues and it is their protein levels that are increased in LSTRA cells and not their relative phosphotyrosine content. However, in a large number of other studies, constitutively enhanced levels of proteins phosphorylated on tyrosine residues have been shown to be due to the presence of an activated tyrosine protein kinase, such as in the case of the K562 cells. Thus, the most likely interpretation of the

results reported in this paper is that the LSTRA cell line also has a constitutively activated tyrosine protein kinase. The most probable candidate for this active enzyme is $pp56^{lck}$, since this protein is greatly elevated in this cell line and the results of analyzing the sites of in vivo phosphorylation of $pp56^{lck}$ in LSTRA cells indicated that some portion of $pp56^{lck}$ is in an active state [7]. These results are especially interesting in light of the fact that overexpression of $pp60^{src}$ does not lead to an active enzyme. It would thus appear that not only is $pp56^{lck}$ overexpressed in LSTRA cells, but it must also be receiving a constant activating signal that results in the elevated level of in vivo activity. Further studies on $pp56^{lck}$ in LSTRA cells should lead to a better understanding of the factors regulating its activity. The presence of numerous phosphotyrosine-containing proteins in LSTRA cells indicates that this cell line should be extremely useful for identifying substrates for tyrosine protein kinases in lymphoid cells.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Life & Health Insurance Medical Research Fund and by NIH grant 38821. We thank Dr Maria Casnellie for her helpful comments on this manuscript.

REFERENCES

[1] Casnellie, J.E., Harrison, M.L., Hellstrom, K.E. and Krebs, E.G. (1982) J. Biol. Chem. 257, 13877–13879.
[2] Casnellie, J.E., Gentry, L.E., Rohrschneider, L.R. and Krebs, E.G. (1984) Proc. Natl. Acad. Sci. USA 81, 6676–6680.
[3] Marth, J.D., Peet, R., Krebs, E.G. and Perlmutter, R.M. (1985) Cell 43, 393–404.
[4] Voronova, A.N. and Sefton, B.M. (1986) Nature 319, 682–685.
[5] Iba, H., Cross, F.R., Garber, E.A. and Hanafusa, H. (1985) Mol. Cell. Biol. 5, 1058–1066.
[6] Coussens, P.M., Cooper, J.A., Hunter, T. and Shalloway, D. (1985) Mol. Cell. Biol. 5, 2753–2763.
[7] Casnellie, J.E. (1987) J. Biol. Chem. 261, in press.
[8] Cartwright, C.A., Kaplan, P.L., Cooper, J.A., Hunter, T. and Eckert, W. (1986) Mol. Cell. Biol. 6, 1562–1670.
[9] Ek, B. and Heldin, C.-H. (1984) J. Biol. Chem. 259, 11145–11152.
[10] Casnellie, J.E. and Lamberts, R.J. (1986) J. Biol. Chem. 261, 4921–4925.
[11] Wang, J.Y.J. (1985) Mol. Cell. Biol. 5, 3640–3643.
[12] Nelson, R.L. and Branton, P.E. (1984) Mol. Cell. Biol. 6, 1003–1012.
[13] Konopka, J.B., Watanabe, S.M. and Witte, O.N. (1984) Cell 37, 1035–1042.
[14] Naldini, L., Stacchini, A., Cirillo, D.M., Aglietta, M., Gavosto, F. and Comoglio, P.M. (1986) Mol. Cell. Biol. 6, 1803–1811.