A Lymphoma Cell Line Expressing Elevated Levels of Tyrosine Protein Kinase Activity*

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The major in vitro substrate for a tyrosine protein kinase in the particular fraction of the lymphoma cell line LSTRA is a protein of approximately 58,000 daltons (pp58). In order to determine if this phosphorylation is unique to the LSTRA cells, the particulate fractions from normal mouse T lymphocytes and another lymphoma cell line, YAC-1, were examined for the presence of pp58 phosphorylation. These two cell types were found to contain this phosphorylation, although the in vitro pp58 phosphorylation is elevated approximately 20-40-fold in the LSTRA cells over that found in T lymphocytes or YAC-1 cells. A comparison was also made of the levels of tyrosine protein kinase activity among these three cell types. Tyrosine protein kinase activity was measured in vitro using an exogenously added synthetic peptide substrate and in vitro by determining cellular phosphoryrosine levels. The results of these determinations indicate that the LSTRA cell line contains an elevated level of tyrosine protein kinase activity.

Previously we reported that a lymphoma cell line, LSTRA, appeared to contain a relatively high level of membrane-associated tyrosine protein kinase activity (1). This conclusion was based on the observation that when the particulate fraction from this cell line was incubated with [γ-32P]ATP, a high level of phosphorylation of a protein, pp58', was obtained. The phosphorylated residue in this protein was identified as phosphotyrosine. The phosphorylation of pp58 is of interest not only because this protein appears to be a very good in vitro substrate for a tyrosine protein kinase, but also because the amino acid sequence around its single site of tyrosine phosphorylation appears to be identical to the amino acid sequences surrounding the sites of tyrosine phosphorylation in two viral tyrosine protein kinases (2).

The LSTRA cell line was originally derived from a mouse infected with Moloney murine leukemia virus (3). Because this virus is thought to transform predominantly cells of the T lymphocyte lineage (4, 5), it was of interest to examine normal mouse T lymphocytes as well as another Moloney leukemia-transformed cell line, YAC-1 (6), for the presence and level of in vitro pp58 phosphorylation. In addition, in order to demonstrate quantitatively the presence of elevated tyrosine protein kinase activity in the LSTRA cells, a comparison was also made among these three cell types of their relative levels of tyrosine protein kinase activity.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The LSTRA cell line was maintained in culture as described (7) with the exception that the media was supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 3 g/liter of sodium bicarbonate. YAC-1 cells were obtained from J. Nepom (Fred Hutchinson Cancer Center, Seattle, WA) and maintained in culture in the above medium devoid of Hepes buffer. Abeloson-MuLV-transformed cells (1881γ) were obtained from N. Rosenberg (Tufts University, Boston, MA) and maintained in culture in the above YAC-1 medium.

Isolation of Mouse Lymphocytes—Lymphocytes were freshly isolated from the spleens of 3-month-old BALB/c mice obtained from an inbred colony maintained by the Fred Hutchinson Cancer Research Center, Seattle, WA. The cells were teased through a stainless steel screen in the above media containing 5% heat-inactivated fetal calf serum. Erythrocytes were removed by hypotonic lysis. Spleen lymphocytes were enriched for T cells by passage of the cell suspension over nylon wool columns (8). Populations were enriched for B lymphocytes by cytotoxic depletion of T lymphocytes with the use of rabbit anti-mouse T cell serum and rabbit complement (Accurate Chemical and Scientific Corp.). The treated cells were depleted of dead T lymphocytes by Ficoll-Isoopaque density centrifugation.

Endogenous Phosphoryrosine Levels—LSTRA and YAC-1 cells (3 × 10^6 cells/ml) were preincubated in phosphate-free RPMI 1640 media (Gibco) supplemented as previously described for the culture of YAC-1 cells and containing 5% heat-inactivated fetal calf serum (undialyzed). After 2 h the cells were centrifuged and resuspended in the above media containing 1 mCi/ml of [32P]orthophosphoric acid (New England Nuclear). The cells were allowed to incubate for 18 h, after which time the protein was extracted and analyzed for phosphoamino acid content as described previously (9), except that excess phenol was removed from the protein fraction by multiple extractions with ether.

Measurements of Tyrosine Protein Kinase Activity with Synthetic Peptide—For these experiments 5 × 10^7 cells were homogenized in 4 ml of 5 mM Hepes, pH 7.4, 1 mM MgCl₂, 5 mM 2-mercaptoethanol. The total particulate fraction was then obtained by centrifuging the homogenates at 150,000 × g for 45 min. The resultant pellet was resuspended in 0.3 ml of 25 mM Hepes, pH 7.4, 5 mM 2-mercaptoethanol and then extracted with 0.3 ml of a buffer containing 2% Triton X-100, 2% deoxycholate, 0.5% sodium dodecyl sulfate, 0.3 M NaCl, 0.2 M Tris, pH 7.2. The material was centrifuged for 2 min in a microfuge and the supernatant collected. The supernatant was then diluted into 25 mM Hepes, pH 7.4, 5 mM 2-mercaptoethanol, 1 mg/ml of bovine serum albumin, and 1% Triton X-100 to give a solution that contained 0.2–0.5 μg/μl of the original cell protein extracted with the detergent buffer.

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The Abbreviations used are: pp58, phosphoprotein of the Rous sarcoma virus gene; R-R-SRC peptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Glu-Gly; Hpes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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The reactions were performed in a 30-µl reaction volume containing 10 mM MgCl₂, 20 µM ZnCl₂ (10), 4 mM p-nitrophenyl phosphate, 100 µM ATP (2000–5000 cpm/pmole), 2–3 µg of detergent-extracted cell protein, and varying concentrations of R-R-SRC peptide. The reactions were run at 30 °C with two concentrations of protein for 1% and 2% min to ensure that the reactions conditions were giving linear rates. The reactions were terminated by the addition of 70 µl of 3.2% trichloroacetic acid followed by 10 µl of 10 mg/ml of bovine γ-globulin. The reactions tubes were centrifuged at room temperature to pellet the protein and the radioactivity incorporated into peptide was determined by spotting 55 µl of the trichloroacetic acid supernatant onto phosphocellulose paper followed by the washing procedure as described previously (1).

In Vitro Phosphorylation and Analysis of pp58—Cell fractions were prepared and phosphorylated as previously described (1). Partial proteolysis of pp58 was performed using the method of Cleveland et al (11).

Analysis of the ³²P-labeled tryptic fragment from pp58 from T lymphocytes and YAC-1 cells was carried out as described previously (2). The comparison with the tryptic fragment from pp60⁵⁷ containing phosphotyrosine was accomplished by using a ³²P-labeled synthetic peptide with the sequence of this tryptic fragment (2).

RESULTS

In Vitro Phosphorylation of pp58 in T Lymphocytes and YAC-1 Cells—Fig. 1 demonstrates that when the particulate fractions from YAC-1 cells and T lymphocytes were incubated with [γ-³²P]ATP, a phosphorylated protein was present in fractions from both cell types that co-migrated with pp58 from LSTRA cells. However, in comparison with the particulate fraction from LSTRA cells, considerably less phosphorylation of pp58 was found in the particulate fraction from YAC-1 cells and T lymphocytes. When gel slices containing pp58 from these three sources were counted, there was 20–40-fold more radioactivity incorporated into pp58 per mg of particulate fraction protein with the fraction from LSTRA cells than with the particulate fractions from the other two cell types. Under the conditions used in Fig. 1 the activity phosphorylating pp58 in all three cell types showed a metal preference of Mn²⁺ over Mg²⁺ (data not shown). As discussed previously (1) this may be due in part to inhibition of phosphatase activity by Mn²⁺. The results in Fig. 1 were obtained under conditions where the phosphorylation of pp58 was nonstoichiometric. Experiments in which the level of phosphorylation is maximal and therefore probably stoichiometric (2–3 min of incubation in the presence of Mn²⁺ and a high concentration of [γ-³²P]ATP (1)) gave results similar to those illustrated in Fig. 1, although in the fractions from YAC-1 cells and T lymphocytes the much lower amount of pp58 phosphorylation made it difficult to distinguish pp58 from background phosphorylation. This result suggests that the actual level of pp58 in LSTRA cells is greater than that in YAC-1 or T lymphocytes.

In order to confirm the identity of the pp58 protein in T lymphocytes and YAC-1 cells the properties of in vitro phosphorylated pp58 from these cells were compared to those of pp58 from LSTRA cells. In vitro phosphorylated LSTRA pp58 contains a single site of tyrosine phosphorylation; the tryptic fragment containing this site co-migrates upon two-dimensional chromatography with the tryptic fragment containing the site of tyrosine phosphorylation in pp60⁵⁷ (2). A similar analysis of the ³²P-labeled tryptic fragment from T lymphocyte pp58 is illustrated in Fig. 2. As with LSTRA pp58, a single major ³²P-labeled tryptic fragment was obtained from T lymphocyte pp58 (A). When this fragment was extracted and analyzed from phosphoamino acid content, all the radioactivity was associated with the tryptic fragment of pp58 containing phosphotyrosine.

FIG. 1. Autoradiogram of the phosphorylated proteins in particulate fractions with LSTRA, YAC-1, and T cells. For this experiment homogenates of the various cell types were initially centrifuged at low speed in order to remove nuclei before preparing the particulate fractions by ultracentrifugation. Phosphorylation reactions contained 10 mM MnCl₂, 0.2 µM [γ-³²P]ATP (3000 Ci/mmol), and were run for 30 s at 30 °C. In the first two lanes the reactions contained 4 µg of LSTRA particulate fraction protein and 15 µg of T lymphocyte particulate fraction protein. In the second two lanes the reactions contained 3 µg of LSTRA particulate fraction protein and 19 µg of YAC-1 particulate fraction protein. The samples were dissolved in sodium dodecyl sulfate solution and subjected to gel electrophoresis. The gels were stained, dried, and autoradiographed by exposing the film to the gels for 20 min. Arrow indicates position of pp58; L, LSTRA; T, T cell; Y, YAC-1.

FIG. 2. Two-dimensional chromatography of the ³²P-labeled tryptic fragment from pp58 from T lymphocytes and the ³²P-labeled synthetic peptide having the sequence of the tryptic fragment from pp60⁵⁷ containing the site of tyrosine phosphorylation. The ³²P-labeled pp58 protein was isolated by gel electrophoresis as in Fig. 1. The protein was then extracted from the gel and subjected to trypsin digestion as described under "Experimental Procedures." The first dimension was electrophoresis with 1% (NH₄)₂CO₃, pH 8.9, for 2 h at 500 V. The second dimension was chromatography in n-butanol/acetic acid/H₂O/pyridine, 75:15:60:60. A, tryptic fragment from pp58. B, tryptic fragment from pp58 + phosphorylated synthetic peptide with sequence of tryptic fragment from pp60⁵⁷.
activity was found associated with phosphotyrosine. In addition, this fragment also co-migrated with the \(^{32}\)P-labeled fragment containing the site of tyrosine phosphorylation in pp60\(^{c-src}\) (B). Further comparison of LSTRA and T lymphocyte pp58 showed that they exhibited identical peptide mapping patterns when electrophoresed in the presence of \(S\). aureus protease (Fig. 3). Results similar to those shown in Figs. 2 and 3 for T lymphocyte pp58 were also obtained with pp58 from YAC-1 cells (data not shown). It is of potential interest that in vitro phosphorylation of pp58 could not be detected in particulate fractions from mouse B lymphocytes, mouse bone marrow cells, or mouse lymphocytes transformed by Abelson murine leukemia virus.\(^2\) In addition, this phosphorylation is completely absent in lymphocytes isolated from the spleens of athymic, nude mice, further identifying the cell type containing pp58 phosphorylation as T lymphocytes.

Levels of Tyrosine Protein Kinase Activity in LSTRA, YAC-1, and T Lymphocytes—LSTRA cells contain a protein kinase that phosphorylates the tyrosine residue in exogenously added R-R-SRC peptide (1). The only phosphorylatable amino acid in this peptide is a tyrosine residue, and kinase activity measurements using this peptide as the substrate provides a means for comparing the relative amounts of tyrosine protein kinase from different sources. Since the amount of pp58 phosphorylation was much greater in the particulate fraction from LSTRA than in the fractions from T lymphocytes and YAC-1, it was of interest to compare the amount of tyrosine protein kinase activity toward the R-R-SRC peptide in the particulate fractions from these three cell types.

In order to perform a meaningful comparison of the activity among these cell types it was necessary to obtain conditions in which peptide phosphorylation was linear with time and protein concentration. Fig. 4 shows a time course of peptide phosphorylation using different amounts of detergent-extracted protein from LSTRA cells. Even at the lowest concentration of protein the reactions were linear for at most 3 min. As shown in Fig. 5, when the incorporation at 2 min was plotted versus protein concentration, it was found that at this time point the incorporation was linear with respect to protein concentration up to 6 \(\mu\)g of protein/assay. Thus, as with other protein kinases (12), it was necessary to use a relatively low concentration of protein in order to obtain linear reactions in crude extracts.

Detergent extracts of total particulate fractions from each of the three cell types were assayed for the presence of kinase activity toward the R-R-SRC peptide. Control experiments showed that extracts from the total particulate fractions yielded more reproducible results and slightly higher total activity than extracts from whole cells. In addition, the soluble fraction was found to contain less than 5% of the activity found in the particulate fraction and all the activity in the particulate fraction was solubilized by detergent. Using incubation conditions under which linear rates of reaction were observed, kinetic constants for the phosphorylation of the R-R-SRC peptide were determined from double reciprocal plots

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2 M. L. Harrison and J. E. Casnellie, unpublished observations.
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TABLE I
Tyrosine protein kinase activity toward RR-SRC peptide

| Cell type   | $K_v$ (mM) | $V_{max}$ (nmol/min/mg) |
|-------------|------------|-------------------------|
| LSTRA       | 2.8        | 7.5                     |
| YAC-1       | 2.4        | 0.5                     |
| T lymphocytes | 3.1        | 9.7                     |

The LSTRA cells were also found to contain elevated levels of tyrosine protein kinase activity when compared with normal mouse T lymphocytes and the YAC-1 cell line. This conclusion is based on in vitro measurements of tyrosine protein kinase activity using a synthetic peptide substrate as well as in vivo determinations of endogenous phosphotyrosine levels. These results confirm our initial observations (1) that the LSTRA cells appeared to express high amounts of tyrosine protein kinase activity. The use of a synthetic peptide that is a specific substrate for tyrosine protein kinases potentially provides a simple procedure for establishing when cells have elevated levels of this activity. This technique yields a direct measure of tyrosine protein kinase activity and eliminates the possibility, for example, that the LSTRA cell line contains elevated levels of phosphotyrosine simply because it contains high amounts of a substrate protein for a tyrosine protein kinase. In addition, this approach allows for comparison of the levels of tyrosine protein kinase activity in situations where it is not practical or not possible to perform measurements of in vivo phosphotyrosine levels. An example of this is T lymphocytes which did not incorporate sufficient quantities of 32P to allow for the measurement of phosphotyrosine levels. It is noteworthy that the specific activity of the tyrosine protein kinase in the total particulate fractions from LSTRA cells, as measured with the synthetic peptide, is nearly equal to the specific activity toward this peptide of the epidermal growth factor receptor kinase in purified plasma membranes from A-451 cells (14). Since the A-451 cells have a greatly elevated level of epidermal growth factor receptor tyrosine protein kinase (15, 16) and this enzyme has a relatively high turnover number for this peptide (14), this comparison provides an additional indication that LSTRA cells contain high levels of a tyrosine protein kinase.

Although the LSTRA and YAC-1 lymphoma cell lines were both induced by infection of mice with Moloney leukemia virus, only the LSTRA cell line has an elevated level of tyrosine protein kinase. Thus, this is not a general property of transformed cells induced by this virus. It is noteworthy that this virus does not encode a tyrosine protein kinase in its genome (17) and consequently could not be the source of the tyrosine protein kinase that is elevated in the LSTRA cells.

The observation that the LSTRA cells have an elevated level of tyrosine protein kinase activity and an elevated level of pp58 phosphorylation in their particulate fraction is intriguing. This protein has a sequence around its site of tyrosine phosphorylation that appears to be identical to the sequence around the sites of tyrosine phosphorylation in two viral tyrosine protein kinases (2). The presence of this sequence homology, encompassing 11 amino acid residues, suggests the possibility that pp58 may also be a tyrosine protein kinase and the in vitro phosphorylation represents an auto phosphorylation reaction. Alternatively pp58 may simply be a substrate for the LSTRA cell tyrosine protein kinase and the elevated level of in vitro phosphorylation is a manifestation of the apparently elevated level of pp58 present in the particulate fraction from these cells. Purification and identification of the tyrosine protein kinase in these cells and studies of its relationship to pp58 will be necessary in order to distinguish these two possibilities.

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