6-1987

Developmental expression of tyrosyl kinase activity in human serum.

Ming-Fong Lin
University of Nebraska Medical Center, mlin@unmc.edu

Joan E. Bailey-Wilson
Louisiana State University

Robert C. Elston
Louisiana State University

Gail M. Clinton
Louisiana State University

Developmental Expression of Tyrosyl Kinase Activity in Human Serum

BY MING-FONG LIN,[1,3] JOAN E. BAILEY-WILSON,[2] ROBERT C. ELSTON[2] AND GAIL M. CLINTON[1,3]

Abstract Tyrosine protein kinases, in addition to their roles as viral transforming proteins and growth factor receptors, have been suggested to have specialized functions in tissue specific processes and in differentiation. High levels of soluble tyrosine kinases have been found in human serum and plasma. To determine if the level of tyrosine kinase activity is developmentally expressed in human serum, we assayed sera from 214 individuals of different ages from newborns to 90 years. We found that serum tyrosine kinase levels are high in newborns and the levels closely parallel skeletal growth until late adolescence. The serum tyrosine kinase levels increase again corresponding to the second and third decades and decline by the fourth decade of life. These studies show that tyrosine kinase levels are developmentally expressed in human serum and delineate the stages in postnatal development when changes in expression occur.

Several growth factor receptors (Heldin and Westermark 1984; Sherr et al. 1985) and oncogene encoded proteins (Bishop 1983; Sefton 1986) have been found to have tyrosine-specific protein phosphorylation activity. Phosphorylation of tyrosine is relatively rare in most normal cells with serine and threonine-specific phosphorylation representing the vast majority of protein phosphorylation events (Hunter and Cooper 1985; Sefton 1986). Because almost all protein tyrosine kinases so far investigated appear to be involved in normal cell proliferation and/or in uncontrolled growth of transformed cells it is important to determine their distribution and expression not only within cells, but also in the whole organism.

In addition to the role of tyrosine protein kinases in cell proliferation, this important group of enzymes has also been suggested to have specialized functions in tissue-specific processes and in differentiation. A possible role in differentiation has been most extensively investigated for pp60src, a cellular tyrosine

[1]Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70112.

[2]Department of Biometry and Genetics, Louisiana State University Medical Center, New Orleans, LA 70112.

[3]Present address: Department of Biochemistry, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201.

Human Biology, June 1987, Vol. 59, No. 3, pp. 549-556

protein kinase which is homologous to the oncogene encoded by Rous sarcoma virus. Cellular pp60src has been found at greatly elevated levels in non-dividing neural tissues of chicks, rats, and humans (Cotton and Brugge 1983; Sorge et al. 1984). Moreover, pp60src is also at elevated levels in nonproliferating cells in the blood particularly in platelets of rabbits and humans (Golden et al. 1986).

Although expression of proteins with tyrosine protein kinase activity has been found to be elevated in specific isolated tissues, it is difficult to determine the pattern of expression of this important group of enzymes during development of the whole organism. Serum represents one of the few specimens which can be used to monitor the expression of developmentally regulated proteins particularly during human growth and development and to evaluate possible changes in expression in human malignant diseases (Gutman 1959; Lewis et al. 1978). We have detected and characterized high levels of soluble tyrosine kinases in human serum and plasma and have determined conditions under which serum kinases can be quantitated without significant interference from serum phosphatases, proteases, ATPases, or kinase inhibitors (Lin et al. 1985). In this communication, by assaying serum enzymes from a large number of individuals, we found that sex, race or several non-malignant disorders did not significantly affect tyrosine kinase levels. Age of the individual, however, had a very significant effect on serum tyrosine kinase levels.

Materials and Methods

Materials. [γ-^{32}P]ATP was purchased from New England Nuclear. Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), Nonidet P-40 (NP-40) were supplied from Sigma. Phosphocellulose paper and 3 MM paper were obtained from Whatman LTD. Sodium vanadate was from Fisher. All other materials were obtained as previously described (Lin et al. 1985).

Serum Preparation. Whole blood (about 5 ml) was removed from apparently healthy volunteers, or hospital patients with non-malignant disorders. Serum was allowed to clot at 4°C overnight. The clot and any remaining unclotted cells were removed by centrifugation at 1,400 xg for 10 min. The supernatant was removed with a pipette and assayed or stored at −70°C in 200 μl aliquots (Lin et al. 1985).

Protein Tyrosine Kinase Assay. Tyrosine kinase assay was performed as previously reported (Lin et al. 1985). Briefly, the substrate, angiotensin II (200 mg), was phosphorylated by serum tyrosine kinases in a 100 μl reaction mixture containing 10 mM Tris, pH 7.0, 10 mM $MnCl_2$, 5 mM dithiothreitol, 0.5% NP-40, 10 μCi [γ-^{32}P]ATP, 25 μM sodium vanadate (ortho), and 1.25 μl of serum at 34°C. An aliquot of 25 μl of the reaction mixture was removed at 2.5,

5, and 10 min and the reaction was terminated by boiling for 3 min. The ^{32}P-angiotensin II was separated from other reaction components by electrophoresis and quantitated by Cerenkov counting.

Total Protein Kinase Assay. The total serum protein kinase activity was quantitated by using endogenous serum proteins as substrates under the same conditions as for the tyrosine protein kinase assays (Lin et al. 1985). After incubation at 2.5, 5, and 10 min at 34°C, a 10 μl aliquot was spotted onto Whatman phosphocellulose paper which was then washed with 0.5% phosphoric acid to remove unincorporated radioactivity. The ^{32}Pi incorporated into protein bound to phosphocellulose paper was quantitated by Cerenkov counting.

Results

Preliminary assays for serum tyrosine kinase activity were conducted using IgG specific for pp60src (Bishop 1983) as the substrate. The levels of anti-src IgG phosphorylating activity measured in sera indicated that the tyrosine kinase levels changed with the age of the individual. To test whether age was significantly associated with tyrosine kinase levels, 214 serum samples from individuals of different ages from newborns to 90 years were assayed. The 214 samples were from 60 White, 136 Black and 18 of unknown race; of these 83 were male, 126 female and 5 of unknown sex. The serum tyrosine kinase activity was assayed for the transfer of ^{32}P from (γ-^{32}P)ATP to the tyrosine residue in angiotensin II peptide under conditions of substrate excess (Lin et al. 1985). Angiotensin II was used as a defined substrate for the determination of tyrosine kinase activity *in vitro* because it can be phosphorylated by several tyrosine kinases (Wong and Goldberg 1983a,b; Lin et al. 1985) and it contains tyrosine but not serine or threonine which are the other amino acids phosphorylated by the known protein kinases. The ^{32}P-labeled angiotensin II was quantitated at 2.5, 5, and 10 min of incubation. To ensure that sera were compared under steady state conditions, only samples that showed increased angiotensin II phosphorylation for 10 min of incubation were used in the age study. (Less than 1% of the serum samples were eliminated on this basis.) Under these reaction conditions, the levels of angiotensin II phosphorylation were not significantly affected by phosphatases, proteases, ATPases, or kinase inhibitors or activators in serum, and were within a range of 5% variation in different aliquots of the same individual serum sample (Lin et al. 1985). In this population study, the enzyme activity of each individual serum sample was obtained by a single determination in a blind mixed experiment.

In these samples, we also determined the level of tyrosine kinases relative

to overall cellular protein kinases. Determinations of the relative levels of tyrosine-specific phosphorylation have been effective in measuring the expression of oncogenes that encode tyrosine kinases and the expression of growth factor receptor tyrosine kinases following the addition of EGF or PDGF to cells (Bishop 1983; Heldin and Westermark 1984). Quantitations of overall protein kinase activity, tyrosine kinase activity and % tyrosine kinase activity are of value in determining whether variability in serum samples occurs for all protein kinases, or just tyrosine-specific kinases. Total serum kinase activity was determined by the levels of endogenous serum protein phosphorylation which included serine, threonine, and tyrosine phosphorylations (Lin et al. 1985). Total protein phosphorylating activity was assayed at 2.5, 5, and 10 min of incubation under conditions of substrate excess. The relative tyrosine kinase levels in the serum samples were calculated by dividing the angiotensin phosphorylating activity by the total serum protein phosphorylating activity measured for 10 min in a kinase assay.

The relative tyrosine kinase levels were plotted according to the age of the serum donor (Fig. 1). The distribution of the points indicated an age related

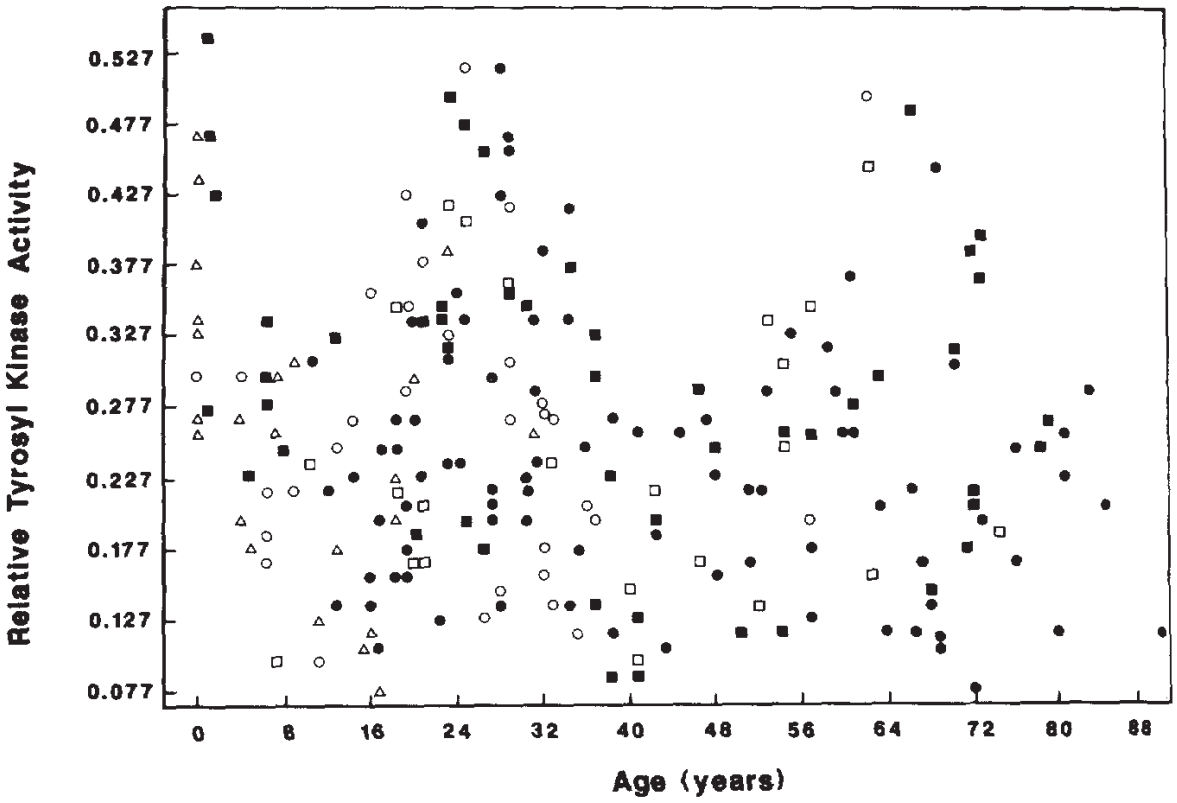

Figure 1. Scatterplot of relative tyrosine kinase levels against age in years for 214 individuals. The tyrosine kinase and the total serum protein kinase activities were quantitated as described in Methods. Levels of phosphate transferred to angiotensin II at 10 min incubation were divided by levels of phosphate transferred to serum proteins at 10 min to determine the relative tyrosine kinase levels. White male (□), Black male (■), White female (○), Black female (●), and unknown sex or race (△).

pattern such that peaks in activity were observed at birth and in the 2nd decade while prominent troughs were seen at about 11 to 15 years and again at about the 4th decade followed by another apparent increase in relative tyrosine kinase levels in the 5th and 6th decades. A qualitatively similar although less pronounced pattern was observed when tyrosine kinase activity alone or when total protein kinase activity was separately plotted (data not shown).

To determine whether there was a statistically significant association of age with relative tyrosine kinase levels, the data points were clustered into different age groups of approximately equal size (18 to 24 data points each), and the group means and their standard errors were calculated for the logarithmically transformed data (Fig. 2). The log of the relative tyrosine kinase activity was used in the statistical analyses to approximately equalize the standard deviations for each group. The pattern demonstrated in Figure 2 is qualitatively similar to the pattern observed when the raw data points were plotted (Fig. 1). Analysis of variance of the data shown in Figure 2 indicated that the differences in levels of relative tyrosine kinase activity among the ten age groups were highly significant ($p<0.003$). In order to further pursue the significance of the difference in relative levels of tyrosine kinase activity among different age groups, the relative tyrosine kinase activity in each age group from low to high were evenly divided into 7 ladder levels. Then, the log of the mean of the lower 4 levels from the age

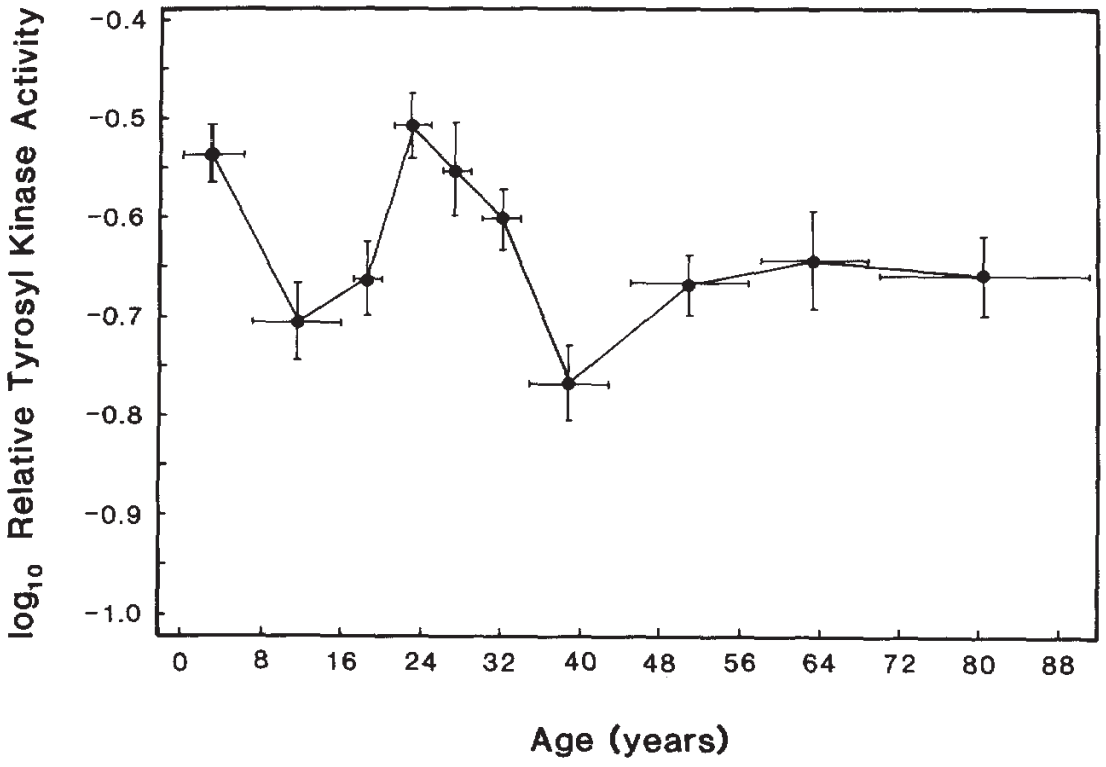

Figure 2. Mean Log_{10} relative tyrosine kinase activity plotted against age in years. Each group contains 18–24 individuals. Horizontal lines indicated that the data points were clustered into different age groups. Vertical lines indicate ± 1 standard error of the mean.

group with high relative tyrosine kinase activity were compared with the log of the mean of the upper 4 levels from the age group with low activity. A significant difference among different age groups in relative tyrosine kinase levels was obtained as well ($p<0.05$). Therefore the pattern observed for the relative tyrosine kinase activity can be explained by an age-related effect. A regression analysis indicated that the relationship between relative tyrosine kinase activity levels and age could not be adequately explained by a linear, quadratic or cubic polynomial. The complex relationship between age and relative tyrosine kinase activity levels is also indicated by the shape of the age curve. Although a similar pattern was observed when the log of the mean tyrosine kinase activity alone or the log of the mean total protein kinase activity was plotted, age was not found to have a significant effect in either case ($p>0.05$).

In addition to age, other factors apparently have an effect on serum kinase activities, since there was variability in the relative tyrosine kinase levels within each age group. Therefore other parameters including race, sex, and health of the individual were tested for their effects on relative tyrosine kinase levels. Sex and race were not found to significantly affect enzyme levels ($p>0.05$). To determine whether the health of the individual played a role, sera were separated into the following seven diagnostic categories: (a) apparently healthy, (b) intrauterine pregnancy, (c) hypertension and high blood pressure, (d) emotional disorders, (e) injury and other tissue trauma, (f) a miscellaneous group of outpatients with nonmalignant disorders which included renal failure, abdominal pain, diabetes mellitus, lead poisoning, and malabsorption, and (g) hospitalized patients with nonmalignant diseases and no diagnostic data due to hospital policy. None of these groups were found to differ significantly in their relative tyrosine kinase levels, nor did they differ in their tyrosine kinase levels, or in their total protein kinase levels. Other unidentified factors such as weight, diet, physical activity, or genetic background may well contribute to the variability observed in the relative tyrosine kinase levels.

Discussion

The effect of age on the relative tyrosine kinase levels in serum strongly suggests that tyrosine kinase activity is developmentally expressed in humans. Changes in expression during tissue-specific differentiation may explain the altered levels of activity in serum. Previous observations suggest that the activities of some serum enzymes may reflect the tissue-specific expression of these enzymes. For example, serum alkaline phosphatase activity of skeletal origin has been found to closely parallel skeletal growth (Gutman 1959). Although the tyrosine kinases in serum may be from normal leakage and release during cell

breakdown as is believed to be the case for alkaline phosphatase, preliminary evidence indicates specific secretion of a novel tyrosine kinase (Lin et al. 1985; Lee et al. in preparation).

The events in human development that correspond to the fluctuations in serum tyrosine kinase levels will be more easily understood when the organ source of the enzyme has been determined. It is possible that the amplified tyrosine kinase levels in cord blood may be from the placenta since elevated expression of the fes oncogene, which encodes for a tyrosyl kinase, has been found in human placenta but not in fetal membranes (Muller et al. 1983). The age curve further indicates that the relative tyrosine kinase activity is high in newborns and then decreases and plateaus through adolescence. Interestingly, this pattern resembles that observed for the rate of skeletal growth (Gutman 1959). The increase in serum activity in the second and third decades is difficult to understand since this group is past the age where most growth and development occur. The slight increase in activity in the fifth and sixth decade may be due to subclinical diseases that are more likely to occur in this age group. The difference in the expression of tyrosine kinase levels in various serum samples from individuals of the same age seems to be a function of biological variation of the individuals, rather than a function of sex and race of the individual. A large variation in the phosphotyrosine levels of different placentas has been observed indicating individual variations in tyrosine kinase activity (Galski et al. 1984).

Although these studies have included measurements of overall tyrosine kinase levels, serum fractionations indicated the presence of only one or a few tyrosine kinase species (Lin, Lee, and Clinton, unpublished observations). Several growth factor receptors, including the EGF, PDGF, and insulin receptors, cannot be detected in serum of any age groups. The age-related differences in relative serum tyrosine kinase activities are apparently not due to the differential expression of the cellular pp60src or other tyrosine kinases from blood cells since the blood cell enzymes can not be detected in serum from any age group (Lin et al. 1985).

Although this study was conducted on serum samples from apparently healthy individuals or patients with non-malignant disorders, in separate studies, we have observed that some type of malignant disorders apparently affect serum tyrosine kinase levels (Lin et al. 1985). Furthermore, sera from tumor-bearing rabbits infected with Rous sarcoma virus had over 5-fold higher levels of pp60src tyrosine kinase activity than did control sera from normal rabbits (Clinton, unpublished observations). These studies indicate that oncogene encoded tyrosine kinases are released from tumors and can be detected at amplified levels in the serum. Further investigations of serum as a source of this important group of enzymes should reveal whether oncogene encoded enzymes or previously unidentified tyrosine kinases are differentially expressed with age in human serum and whether their expression may be diagnostic for human cancer.

Acknowledgements We thank the Clinical Chemistry Lab at Charity Hospital and Dr. Theodore Thurmon of the Pediatrics Department at LSU for serum samples, Dr. Herman Opperman for the gift of anti-pp60src serum, Ms. Tina Tan for excellent technical assistance, Dr. Jean Carr for helpful discussions, and Ms. Kim Tervalon for excellent secretarial support. This work was supported by USPHS grants from the National Cancer Institute CA-34517 and CA-39641 to GMC and CA 28198-05 to RCE.

Received 2 July 1986; revision received 25 November 1986.

Literature Cited

BISHOP, J.M. 1983 Cellular oncogenes and retroviruses. Ann. Rev. Biochem. **52**:301–354.

COTTON, P.C. AND J.S. BRUGGE 1983 Neural tissues express high levels of the cellular src gene product pp60c-src. Mol. Cell. Biol. **3**:1157–1162.

GALSKI, H., N.D. GROOT, J. ILAN AND A.A. HOCHBERG 1984 Phosphorylation of tyrosine in cultured human placenta. Biochim. Biophys. Acta, **804**:125–131.

GOLDEN, A., S.P. NEMETH AND J.S. BRUGGE 1986 Blood platelets express high levels of the pp60c-src-specific tyrosine kinase activity. Proc. Natl. Acad. Sci. USA **83**:852–856.

GUTMAN, A.B. 1959 Serum alkaline phosphatase activity in diseases of the skeletal and hepatobiliary systems. Am. J. Med. **27**:875–901.

HELDIN, C.-H. AND B. WESTERMARK 1984 Growth factors: Mechanism of action and relation to oncogenes. Cell, **37**:9–20.

HUNTER, T. AND J.A. COOPER 1985 Protein-tyrosine kinases. Ann. Rev. Biochem. **54**:897–930.

LEWIS, V.M., J.J. TWOMEY, P. BEALMEAR, G. GOLDSTEIN AND R.A. GOOD 1978 Age, thymic involution, and circulating thymic hormone activity. J. Clin. Endocrinol. Metab. **47**:145–150.

LIN, M.-F., P. LEE AND G.M. CLINTON 1985 Characterization of tyrosyl kinase activity in human serum. J. Biol. Chem. **260**:1582–1587.

MULLER, R., J.M. TREMBLAY, E.D. ADAMSON AND I.M. VERMA 1983 Tissue and cell type-specific expression of two human c-onc genes. Nature, **304**:454–456.

SEFTON, B.M. 1986 The viral tyrosine protein kinases. Curr. Top. Microbiol. Immunol. **123**:39–72.

SHERR, C.J., C.W. RETTENMIER, R. SACCA, M.F. ROUSSEL, A.T. LOOK AND E.R. STANLEY 1985 The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell, **41**:665–676.

SORGE, L.K., B.T. LEVY AND P.F. MANESS 1984 pp60c-src is developmentally regulated in the neural retina. Cell, **36**:249–257.

WONG, T.W. AND A.R. GOLDBERG 1983a *In vitro* phosphorylation of angiotensin analogs by tyrosyl protein kinases. J. Biol. Chem. **258**:1022–1026.

WONG, T.W. AND A.R. GOLDBERG 1983b Tyrosyl protein kinases in normal rat liver: identification and partial characterization. Proc. Natl. Acad. Sci. USA **80**:2529–2533.