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Identification and Characterization of Tyrosine Kinase Activity Associated With Mitochondrial Outer Membrane in Sarcoma 180 Cells

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Tyrosine protein kinase activity has been detected in the mitochondrial fraction purified from sarcoma 180 tumor cells. Following hypotonic disruption of mitochondria, tyrosine kinase activity appeared to cosediment with monoamine oxidase, marker enzyme of mitochondrial outer membrane; meanwhile, serine and threonine kinases were found to be associated with the inner membrane and matrix of mitochondria. Mitochondrial tyrosine kinase(s) showed thermosensitivity and Mn^{2+} dependence, useful properties for its characterization and separation from tyrosine kinases associated with other particulate fractions and from serine and threonine kinases associated with mitochondria. Following in vitro incubation of mitochondria with labelled ATP as substrate and analysis by PAGE, a complex pattern of phosphotyrosine containing proteins with a major band of 50–55 kilodaltons resulted.

Key words: tyrosine kinase, phosphorylation, mitochondria

In the last few years it has been reported that at least four receptors for growth factors are endowed with tyrosine kinase activity [1]; as a consequence, plasma membrane has been considered the main subcellular location of such enzyme activity. Indeed, few reports have dealt with the localization of tyrosine kinase activities on other subcellular particulate fractions apart from plasma membrane [2].

Tyrosine kinase activity has also been found to reside in oncogene products of transformed cells [3] and to be associated with plasma membranes of neoplastic cells, including a hepatoma and an ascites cell line [4]; furthermore, it has been reported that its activity increases following transformation by retroviruses [5].

In a number of normal and tumor cells we have described the association of a tyrosine kinase activity with a postnuclear particulate cell fraction sedimenting at

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12,000g [6]; this activity was several fold higher in neoplastic or virus-transformed cells than in normal ones. In cultured diploid human fibroblasts we have recently demonstrated by an in vitro assay that this tyrosine kinase activity is associated with mitochondria and increased severalfold when the cells were pushed to quiescence [7]. The aim of this work, then, was to further characterize this kinase activity, to define its precise mitochondrial location, and to analyze its endogenous substrates. In this study we have used a sarcoma ascites tumor cell, which, among several normal and neoplastic cellular models tested, appeared to be endowed with the highest mitochondrial tyrosine kinase activity [6].

MATERIALS AND METHODS

Cells

Experiments were performed on sarcoma 180 ascites tumor cells which were maintained in Balb/c female mice by weekly transplantation of 2–3 $\times$ 10^6 cells. Tumor-bearing animals with growths between 5 and 7 days were used. Approximately 10^9 cells were removed from the peritoneal cavity by aspiration and washed free of ascitic fluid using an isotonic buffer.

Purification and Characterization of Submitochondrial Particles

Mitochondria were purified from sarcoma 180 cells according to the procedure previously described [8] with a few modifications. The washed cells were exposed for 10 min to a hyposmotic shock in cold HE solution (10 mM HEPES, pH 7.6; 2 mM EDTA), and the isotonicity of the suspension was reconstituted by addition of a large volume of H solution (0.22 M mannitol; 0.07 M sucrose; 2 mM HEPES, pH 7.8; 0.1% albumin). The cells were gently lysed by means of a Dounce homogenizer (ten strokes or less). Unbroken cells, cell debris, and nuclei were then removed by centrifuging at 2,000g. The postnuclear supernatant thus obtained was centrifuged at 12,000g for 20 min. The 12,000g supernatant was further centrifuged at 120,000g for 30 min; and the resulting pellet, enriched with membranous subcellular structures other than mitochondria, was referred to as postmitochondrial particulate fraction (PMPF). The 12,000g pellet was resuspended in HSE solution (0.25 M sucrose; 10 mM HEPES, pH 7.6; 2 mM EDTA; and 100 U/ml aprotinine) and centrifuged again at 12,000g. This step was repeated twice. The final pellet was resuspended and loaded on the top of a 0.6–1.8 M continuous sucrose gradient and centrifuged at 120,000g for 3 hr in an SW 27 Beckman-Spinco rotor. At the end of the run a large band corresponding to a density range of 1.17–1.19 g/cm^3 was clearly visible in the gradient. This band, corresponding to the mitochondrial fraction, was collected, diluted in HSE solution and centrifuged at 120,000g for 30 min.

When the separation of the submitochondrial structures was pursued, the procedure described by Sottocasa et al [9] was followed with a few modifications. Briefly, the 120,000g pellet was resuspended in a hyposmotic solution of 10 mM Tris-phosphate buffer, pH 7.5. After standing at 0°C for 15 min, a solution containing 1.8 M sucrose and 2 mM $MgSO_4$ was added to reach a final concentration of 0.25 M sucrose. The resulting suspension was layered on the top of a 0.6–1.8 M linear sucrose gradient and centrifuged at 120,000g for 3 hr. After the run, the large visible band corresponding to a density range of 1.16–1.21 g/cm^3 was eluted into several fractions. Each of these fractions was diluted with an appropriate volume of HE

solution, divided into two aliquots, and centrifuged at 120,000g for 30 min. One of the pellets thus obtained after resuspension in the appropriate buffer was tested for marker enzyme analysis; the other pellet after resuspension in an appropriate volume of HT medium (10 mM HEPES, pH 7.6; 5 mM 2-mercaptoethanol; 1% Triton X-100; and 100 U/ml aprotinine) was used for assaying kinase activities. All the operations described in this paragraph were performed at 4°C.

Marker Enzyme Assays

To determine the grade of purity of the mitochondrial preparations, the activities of 5′-nucleotidase, acid phosphatase, glucose-6-phosphatase, succinate dehydrogenase, malate dehydrogenase, and monoamine oxidase were measured in the mitochondrial fraction, collected from the sucrose gradient, and in the PMPF [as described in 9-14]. Protein content of the PMPF and submitochondrial fractions was determined according to the method of Lowry et al [15].

Protein Kinase Assay and Polyacrylamide Gel Electrophoresis (PAGE) Analysis

The reaction mixture contained 10 mM HEPES (pH 7.6), 2 mM magnesium acetate, 10 mM $MnCl_2$, and 2 μCi [γ-^{32}P] ATP (3,000 Ci/mmol, Amersham, Buckshire, U.K.). Reaction was started by adding 10 μl of the mitochondrial preparation (25 μg of proteins) in a final volume of 50 μl and carried out at 30°C for 2 min (condition approaching initial velocity reaction). Assay was terminated by adding an appropriate volume of sample buffer (2% SDS; 62.5 mM Tris-HCl, pH 6.8; 2% glycerol; 0.5% 2-mercaptoethanol; and 0.004% bromophenol blue). After heating for 3 min in a boiling water bath the samples were then subjected to electrophoresis on SDS-polyacrylamide slab gels using a standard apparatus (BIORAD, Richmond, CA). Equal amounts of proteins were loaded onto the gels. Proteins were separated on 10% (w/v) acrylamide slab gels in a discontinuous buffer system as described by Laemmli [16] with a constant current of 25 mA for approximately 5 hr. The gels were then fixed, stained with Coomassie blue, dried, and exposed to Kodak X-omat X-ray film for 1 day; the film was then developed. When required, phosphoproteins were eluted and processed for phosphoamino acid analysis following the procedure described in [17].

Phosphoamino Acid Analysis

To determine phosphoamino acid levels in mitochondria or in submitochondrial fractions, the phosphorylation reaction, carried out as described above, was stopped by adding an appropriate volume of HCl (final concentration 6 N). The acid hydrolysis of proteins was carried out at 110° C for 2 h in vacuum sealed tubes. The HCl was removed by evaporation and the residues were dissolved in a marker mixture containing phosphoserine, phosphothreonine, and phosphotyrosine (1 mg/ml each) and analyzed by bidirectional paper electrophoresis as previously described [18]. A 50-μl aliquot of the hydrolysate was spotted on 3 MM Whatman paper (100 μm) and subjected to electrophoresis in the first direction at pH 1.9 (acetic acid/formic acid/H_2O, 150:25:825) for 2 hr at 4 kV and in the second direction at pH 3.5 (acetic acid/pyridine/H_2O, 50:5:945) for 1 hr at 2.4 kV. Markers were detected by staining with ninhydrin, and to identify the ^{32}P-labelled phosphoamino acids, an autoradiogram was made by exposing the electropherogram to Kodak X-omat X-ray film for 1 day.

The incorporation of ^{32}P was quantified by cutting out the appropriate paper pieces corresponding to the three phosphoamino acid spots, placing them directly into scintillation fluid, and measuring the radioactivity by liquid scintillation spectrometry.

RESULTS

Association of Serine, Threonine, and Tyrosine Kinase Activities With Mitochondria

The degree of purification of the mitochondrial fraction (MF) was evaluated by testing marker enzymes for different cellular organelles. The results of this analysis were plotted in the diagrammatic fashion adopted by De Duve et al [19]. As shown in Figure 1a, the activities of marker enzymes specific for plasma membranes (5′-nucleotidase), microsomes (glucose-6-phosphatase), and lysosomes (acid phosphatase) were slightly detected in the mitochondrial fraction, almost all the activity of these enzymes being associated with the postmitochondrial particulate fraction (PMPF). On the contrary, the activities of two mitochondrial marker enzymes such as succinate dehydrogenase and monoamine oxidase (see Fig. 1b,c) were enriched 20–30-fold in the MF in comparison to PMPF. It should be noted that a similar distribution pattern has been obtained by using malate dehydrogenase as a mitochondrial marker enzyme (result not presented). In the same figure, the distribution patterns of serine (Fig. 1d), threonine (Fig. 1e), and tyrosine kinase activity (Fig. 1f) are presented: as shown, the relative specific activities of the three kinases resulted in curves almost superimposable on one another and corresponded to 2.3%, 2.7%, and 2.2%, respectively, of the related PMPF kinase activity.

Location of Kinase Activities in the Submitochondrial Structures

The MF purified by sucrose gradient equilibrium sedimentation was pelleted by centrifugation and then exposed to an osmotic shock with the aim of separating submitochondrial structures. The resulting suspension was then analyzed following a further sucrose gradient sedimentation. Several fractions of increasing density were collected from the gradient, and activities of marker enzymes specific for mitochondrial outer membranes, inner membranes, and matrix as well as activities of serine, threonine, and tyrosine kinases were measured. The results of this enzymatic analysis are presented in Figure 2. As shown in this figure, succinate and malate dehydrogenases (marker enzymes of inner membranes and matrix) were found to sediment together, their maximum activity having a peak around density of 1.195 g/cm^3, whereas monoamine oxidase (marker enzyme for outer membranes) sedimented at density of 1.162 g/cm^3. This result indicated a definite separation between mitochondrial outer membranes and mitoplasts. The activity of serine kinase appeared to reach its maximum with the fraction sedimenting at a density of 1.195 g/cm^3, in comparison to that of tyrosine kinase, which sedimented at a density of 1.162 g/cm^3. The activity of threonine kinase, however, did not peak as sharply as the other kinases, because of its broad distribution in several fractions; it should also be noted that only a small percentage of its activity was found to be associated with the lighter fractions. The possible contamination of the submitochondrial structures by other subcellular organelles sedimenting around the same density was ruled out by the absence of 5′-nucleotidase, glucose-6-phosphatase, and acid phosphatase activities in all the density gradient fractions tested.

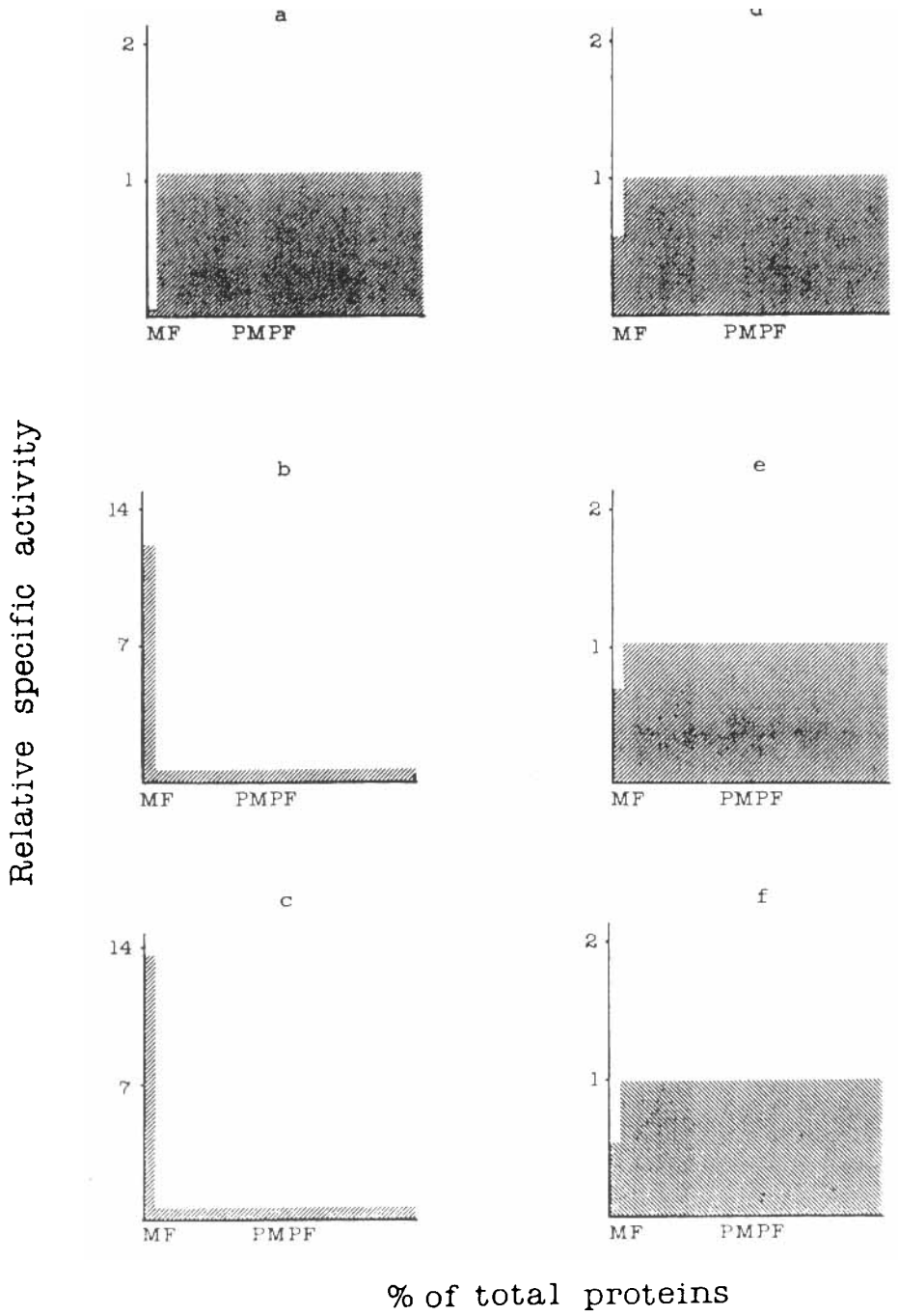


Fig. 1. The de Duve plot of the distribution patterns of marker enzymes and protein kinases for mitochondrial fraction (MF) and postmitochondrial particulate fraction (PMPF). **a:** 5′- nucleotidase, acid phosphatase, glucose-6-phosphatase. **b:** Succinate dehydrogenase. **c:** Monoamine oxidase. **d:** Serine kinase. **e:** Threonine kinase. **f:** tyrosine kinase. Total protein corresponded to the sum of the protein contents present in MF and PMPF. Relative specific activity corresponded to the percentage of total enzyme activity present in that fraction divided by the percentage of total protein present in the same fraction. The values shown in panels a–c are the mean of seven independent determinations. The values shown in panels d–f are the mean of three independent determinations.

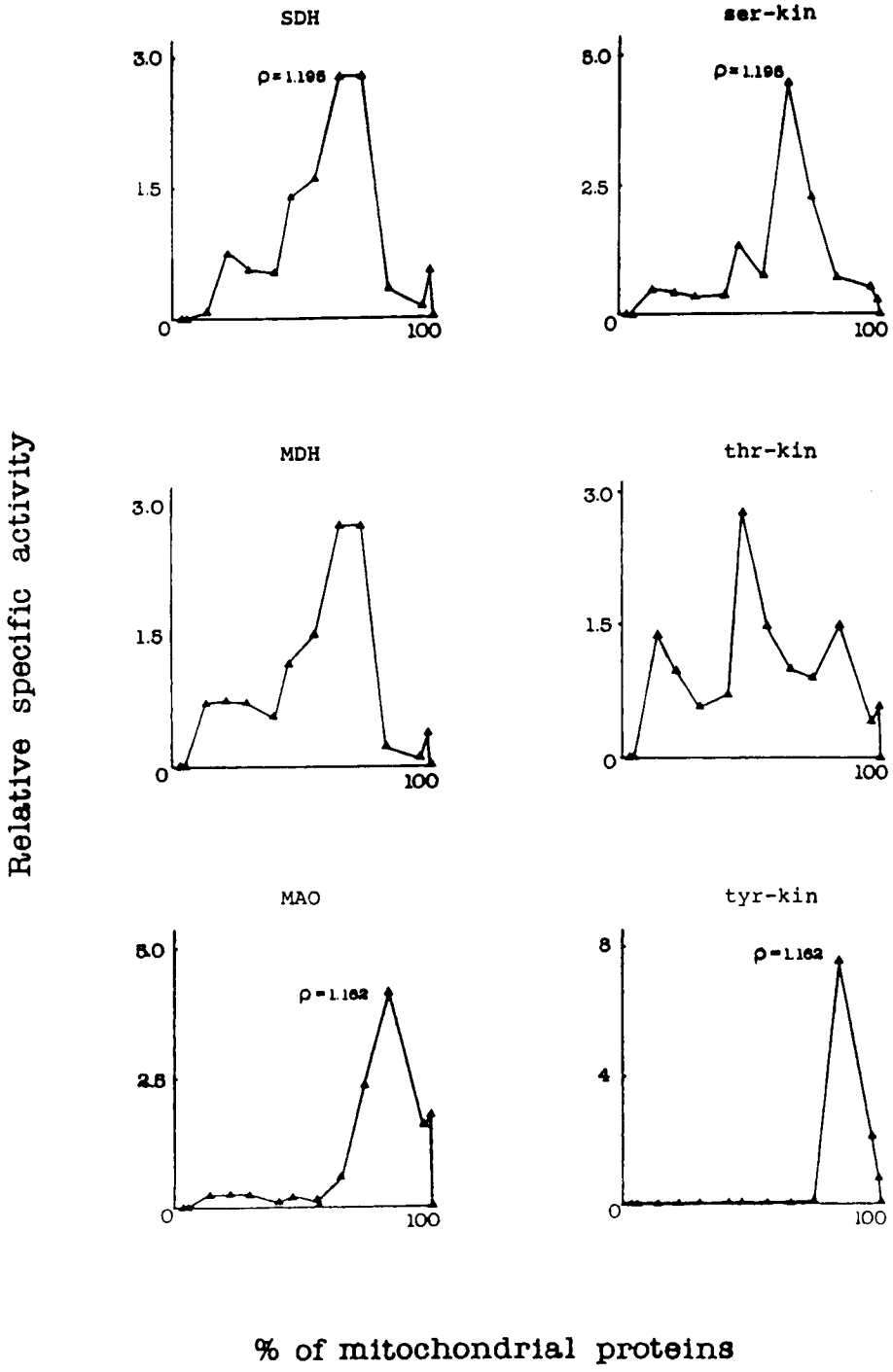
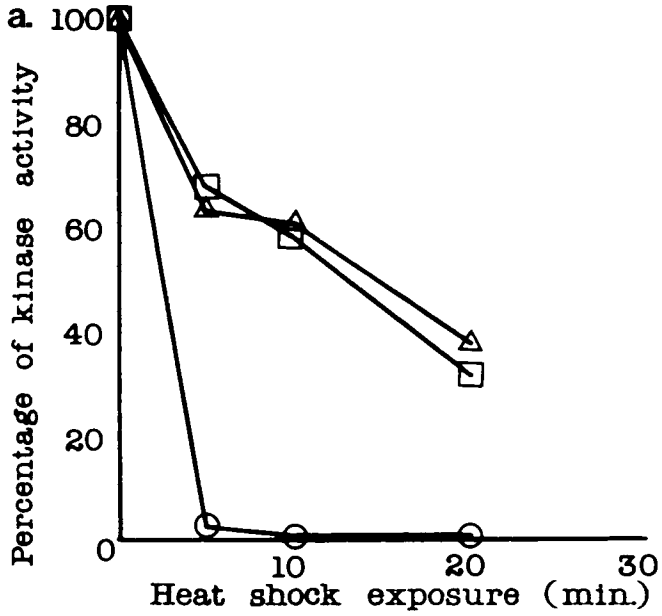


Fig. 2. The de Duve plot of the distribution pattern of intramitochondrial location marker enzymes and of serine, threonine, and tyrosine kinases. Ser-kin, serine kinase; thr-kin, threonine kinase; tyr-kin, tyrosine kinase; SDH, succinate dehydrogenase; MDH, malate dehydrogenase; MAO, monoamine oxidase. The values shown in panels SDH, MDH, and MAO are the mean of seven independent determinations. The values shown in panels ser-kin, thr-kin, and tyr-kin are the mean of three independent determinations.

Characterization of Mitochondrial Tyrosine Kinase Activity

Temperature dependence. Figure 3 presents the results of experiments carried out to evaluate the thermosensitivity of the phosphorylation reaction of mitochondrial proteins at serine, threonine, and tyrosine residues. As shown in Figure 3a, tyrosine phosphorylation appeared to be extremely sensitive to a short (5 min) exposure of mitochondria at 43°C, whereas a substantial portion of phosphorylation at serine and threonine residues still occurred after longer heat exposure (20 min). Figure 3b shows the electropherogram of phosphorylated amino acids extracted from mitochondria exposed or not to a heat shock (10 min at 43°C) before the phosphory-



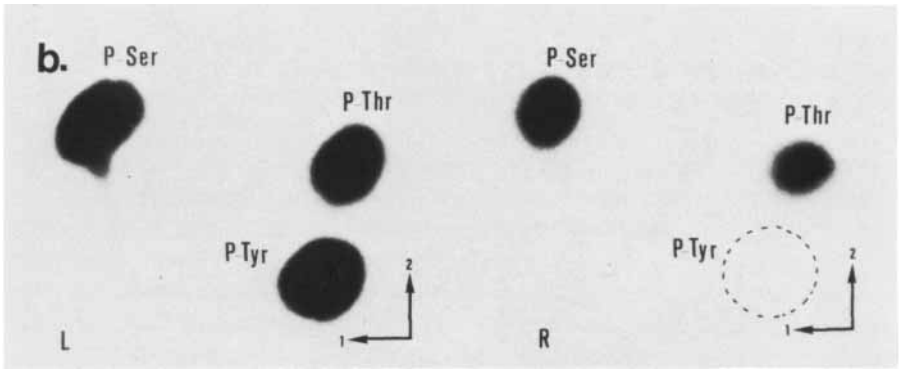


Fig. 3. Thermosensitivity of the phosphorylation reaction of mitochondrial proteins. **a:** Effect of heat (43°C) exposure on the phosphorylation reaction at serine (□), threonine (△), and tyrosine (○) residues of mitochondrial proteins. **b:** Bidirectional electrophoresis of amino acids phosphorylated by protein kinase activities before (L) and after (R) a heat-shock exposure (10 min) of the mitochondrial fraction. Arrows on the autoradiogram indicate first and second directions of electrophoretic migrations from the origin. The values shown in panel a are the mean of three determinations with standard deviations of less than 10%.

lation reaction. As shown, the spot corresponding to phosphotyrosine did not appear in the electropherogram of the phosphoamino acids extracted from heat-shocked mitochondria. It should also be noted that tyrosine phosphorylation in the PMPF (cf. Table I) did not appear to be significantly affected by a short heat exposure (5 min at 43°C).

Mn^2 dependence. In Table II the Mn^{2+} dependence of tyrosine kinase associated with the MF is presented. As shown in this table, the withdrawal of Mn^{2+} from the reaction medium did affect the activity of mitochondrial tyrosine kinase in a highly significant way, whereas no substantial deviation from control values occurred for the tyrosine kinase activity associated with the PMPF.

Apparent MW of the target proteins. Purified mitochondria were exposed to an osmotic shock and sedimented to equilibrium on sucrose gradient as previously described. The resulting submitochondrial fractions were collected from the gradient, and the kinase activity associated with each fraction was tested before and after a heat shock. The phosphorylation pattern of the endogenous substrates is presented in Figure 4. As shown in this figure, in the heavy fractions, which contained serine and threonine kinase activities but were devoid of tyrosine kinase activity, a phosphorylated band with apparent molecular weight of 42 kilodaltons (kD) occurred. The light fractions, however, which were endowed with tyrosine kinase activity, presented a complex pattern of phosphorylated endogenous substrates, with a prominent broad band in the 50–55-kD region. In the same autoradiogram is shown the heat resistance and the thermosensitivity of the phosphorylation reaction of the 42- and 50–55-kD bands, respectively (cf. Fig. 4, lanes 7 or 8 and 9). Analysis of the phosphorylated amino acids present in the bands and eluted from the gel, indicated a large presence (97%) of phosphoserine with a concomitant small amount of phosphothreonine in the 42-kD band, whereas phosphotyrosine appeared to be the predominant residue (90%) of the 50–55-kD band (see Table III).

TABLE I. Thermosensitivity of Tyrosine Kinases*

Cell fractions	Control	Heat shock
MF	57,621 ± 5,112	3,101 ± 281
PMPF	98,619 ± 7,112	82,108 ± 1,289

*The phosphorylation reaction before and after a heat shock (10 min at 43°C) was performed at 30°C. Identical amounts of proteins were incubated, hydrolyzed, and subjected to bidirectional electrophoresis to separate phosphorylated amino acids as described in Materials and Methods. The values shown ± SD correspond to the cpm present on each spot of phosphotyrosine as revealed by liquid scintillation spectrometry.

TABLE II. Mn^{2+} Dependence of Mitochondrial Tyrosine Kinase*

Cell fraction	$-Mn^{2+}$	$+Mn^{2+}$
MF	7,202 ± 891	57,621 ± 3,212
PMPF	96,108 ± 8,915	98,619 ± 2,718

*Tyrosine protein kinase reaction was carried out as described in Materials and Methods except that 10 mM $MnCl_2$ was present only where indicated. The values shown ± SD correspond to each spot of phosphotyrosine, after separation by bidirectional electrophoresis, as revealed by liquid scintillation spectrometry.

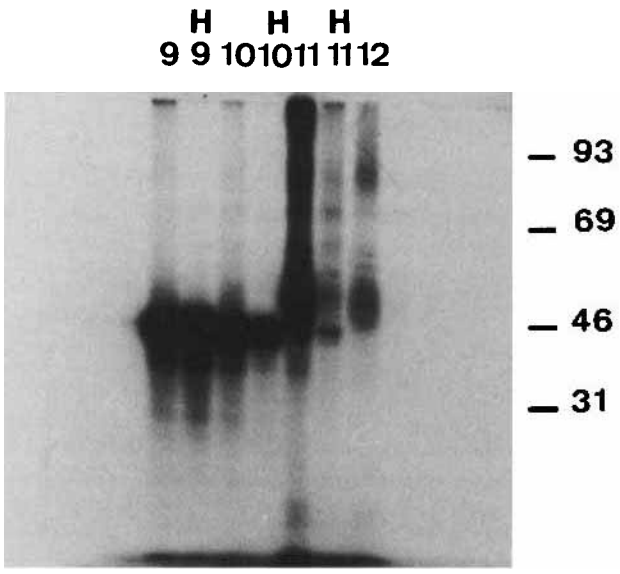


Fig. 4. PAGE analysis of endogenous protein substrates phosphorylated in vitro. Submitochondrial fractions purified by sucrose gradient sedimentation as described in Materials and Methods were exposed (H) or not exposed to a heat shock (5 min at 43°C) and then assayed for protein kinase activity. The endogenous reaction products were analyzed by PAGE under standard conditions. The molecular weights of the phosphorylated substrates were calculated by comparing their relative mobilities with those of comigrated protein standards.

TABLE III. Phosphoamino Acid Analysis of the 42-kD and 50–55-kD Bands*

Bands (kD)	Phosphoserine	Phosphothreonine	Phosphotyrosine
42	97	3	—
50–55	7	4	89

*Phosphoamino acid analysis of the 42-kD and 50–55-kD bands eluted from the gel was carried out as described in Materials and Methods. Values shown indicate the relative percentage of phosphoserine, phosphothreonine, and phosphotyrosine in the sample.

TABLE IV. Apparent Km Values for ATP and GTP of Mitochondrial Protein Kinases in Sarcoma 180 Ascites Tumor Cells*

Substrate	Serine kinase (μM)	Threonine kinase (μM)	Tyrosine kinase (μM)
ATP	66	72	476
GTP	36	48	381

*Initial rates (2 min) of serine, threonine, or tyrosine kinase activity were determined under conditions described in Materials and Methods. Data were analyzed by the Eadie-Hofstee method. The range of substrate concentrations tested was 0.025–2 mM.

Apparent kinetic parameters. The source of the phosphate group for the phosphorylation reaction catalyzed by the mitochondrial kinases has been investigated by using ATP and GTP as phosphate donors. As shown in Table IV, the apparent Km values of serine, threonine, and tyrosine kinases did not appear to be significantly affected when GTP was used as donor instead of ATP. An additional but significant

result of these analyses is the marked difference of the tyrosine kinase apparent Km in comparison to that of serine and threonine kinases.

Effect of orthovanadate. Data not presented here have indicated that tyrosine phosphorylation in the light fraction was not significantly modified by the presence of orthovanadate, a well-known specific inhibitor of phosphotyrosyl protein phosphatase [20]. This result suggests that in the purified submitochondrial structures a phosphotyrosyl phosphatase activity is absent or inactive under conditions used in our assay.

DISCUSSION

The results presented in this work indicate the association of tyrosine kinase activity, as determined by an in vitro phosphorylation reaction, with a submitochondrial fraction obtained from sarcoma 180 ascite cells and purified by isopycnic sedimentation. By specific marker enzyme analysis, evidence has been presented of the association of this tyrosine kinase activity with the external mitochondrial structures. Indeed, the lighter fractions of the submitochondrial structures isolated by differential centrifugation, following an osmotic shock procedure, were endowed with tyrosine kinase activity as well as monoamine oxidase activity. Monoamine oxidase is a well-established marker enzyme for the mitochondrial outer membrane [9]. Serine and threonine kinase activities sedimented together with succinate and malate dehydrogenases, well-known marker enzymes for mitochondrial inner membranes and matrix, respectively [9].

The tyrosine kinase activity associated with mitochondria presented a thermosensitivity higher than that of the other two kinases associated with the mitochondrial fraction. Furthermore, a marked difference between mitochondrial tyrosine and serine or threonine kinases in the apparent Km values for ATP or GTP as phosphate donors has been found. These data suggest marked biophysical and biochemical differences between mitochondrial tyrosine and threonine or serine kinases. It should also be noted that tyrosine kinase associated with PMPF has shown a thermostability higher than that revealed by mitochondrial tyrosine kinase. Taking into account the specific Mn^{2+} dependence of the mitochondrial tyrosine kinase, evidence suggests that mitochondria- and PMPF-associated tyrosine kinases are different enzymes.

By using PAGE analysis of the phosphorylated substrates, we have identified a major substrate of 42 kD for serine and threonine kinases and a complex pattern of phosphotyrosine containing substrates for tyrosine kinase with a major band of 50–55 kD. It should be noted that similar phosphotyrosine-containing protein bands have been reported in high-speed-sedimenting particulate fractions, which included mitochondria, isolated from the lymphoma cell line LSTRA [21], rat spleen [22], human lymphocytes [23], and murine Leydig tumor cells [24]. However, any relationship between the 50–55-kD labelled band described in this work and the labelled bands reported in the above-mentioned cell models are at present unknown.

Protein kinases have previously been isolated from rat liver mitochondria and reported to phosphorylate serine residues [25]; in addition, a protein kinase activity associated with the inner membrane of mouse liver mitochondria has been described [26]. In the latter work, evidence has been offered on the presence of a protein kinase activity within the mitochondrial outer membrane. However, the author has not attempted the separation of the phosphorylated residues; therefore, a similarity be-

tween protein kinase associated with the outer membrane of mouse liver mitochondria and the tyrosine kinase associated with outer membrane of sarcoma 180 tumor cells described here should be considered only as tentative.

Serine and threonine protein kinases have recently been purified and characterized from bovine heart mitochondrial membranes, and it has been suggested that these enzymes are not located on the membrane surface of the mitochondria but should be considered as intrinsic membrane proteins [27]. However, because of the differences in the procedures used and cell models adopted, any possible correlation between the enzyme activities mentioned in this paragraph and threonine or serine kinase activities associated with the mitoplast structures of sarcoma 180 ascites tumor cells is not allowed at present.

On the basis of the evidence presented in this work, we propose that protein phosphorylation in mitochondria involves at least two different enzymes or enzymatic systems. The first phosphorylates tyrosine residues, is heat-sensitive, is endowed with a high Km value for ATP as substrate, and is associated with the outer mitochondrial membrane; the second phosphorylates serine and threonine residues, is thermoresistant, presents low Km values, and is localized in the mitochondrial internal structures (mitoplast). In addition, tyrosine kinase activity associated with the mitochondrial outer membrane appears to be different from tyrosine kinase activity associated with PMPF.

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REFERENCES

1. Heldin CH, Westermark B: Cell 378:9, 1984.
2. Swarup G, Dasgupta JD, Garbers DL: Adv Enzyme Regul 22:267, 1984.
3. Bishop JM: Cell 42:23, 1985.
4. Usui H, Yoshokawa K, Imazu M, Tsukamoto H, Takeda M: FEBS Lett 184:60, 1985.
5. Sefton BM, Hunter T, Beemon K, Eckhart W: Cell 20:807, 1980.
6. Montagnier L, Chamaret S, Dauguet C: CR Acad Sci 295T:375, 1982.
7. Piedimonte G, Silvotti L, Chamaret S, Borghetti AF, Montagnier L: J Cell Biochem 32:113, 1986.
8. Pedersen PL, Greenwalt JM, Reynafarje B, Hullihen J, Decker GL, Soper JW, Bustamente E: Methods Cell Biol 20:411, 1979.
9. Sottocasa GL, Kuylenstierna B, Ernster L, Bergstrand A: In Estabrok RW, Pullman ME (eds): "Methods in Enzymology," Vol 10. 1967, p 448.
10. Michell RH, Hawthorne JN: Biochem Biophys Res Comm 21:33, 1965.
11. Bergmeyer HY: "Methods in Enzymatic Analysis." New York: Academic Press, 1970, p 613.
12. Ray TK: Biochim Biophys Acta 196:1, 1970.
13. Earl DCM, Korner A: Biochem J 94:721, 1965.
14. Tabor CW, Tabor H, Rosenthal SM: J Biol Chem 208:654, 1954.
15. Lowry DH, Rosebrough NJ, Farr AL, Randall RY: J Biol Chem 193, 265, 1951.
16. Laemmli UK: Nature 206:680, 1970.
17. Beemon K, Hunter T: J Virol 28:551, 1978.
18. Ushiro H, Cohen S: J Biol Chem 255:8363, 1980.
19. de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F: Biochem J 60:604, 1955.

20. Swarup G, Speeg KV, Cohen S, Garbers DL: J Biol Chem 257:7298, 1982.
21. Casnellie JE, Harrison ML, Pike LJ, Hellstrom KE, Krebs EG: Proc Natl Acad Sci USA 79:282, 1983.
22. Swarup G, Dasgupta JD, Garbers DL: J Biol Chem 258:10341, 1983.
23. Piga A, Wickremasinghe RG, Taheri MR, Yaxley JC, Hoffbrand AV: Exp Cell Res 159:103, 1985.
24. Dangott LJ, Puett D, Melner MH: Biochim Biophys Acta 886:187, 1986.
25. Clari G, Pinna LA, Moret V: Biochim Biophys Acta 451:484, 1976.
26. Vardanis A: J Biol Chem 252:807, 1977.
27. Kitagawa Y, Racker E: J Biol Chem 257:4547, 1982.