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Association of Tyrosine Protein Kinase Activity With Mitochondria in Human Fibroblasts

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A tyrosine protein kinase activity has been detected in the mitochondrial fraction purified from human fibroblasts. By enzymatic and sedimentation analysis this activity appeared to be localized in the mitochondrial outer membrane. Mitochondrial tyrosine phosphorylation was strictly dependent on the presence of Mn^{2+} ions. An inverse relationship between cell proliferation and mitochondrial protein phosphorylation on tyrosine residues has been found: a marked increase in the mitochondrial tyrosine kinase activity occurred when a significant reduction in the growth rate followed serum step-down. In mitochondria purified from resting cells, a protein band with apparent molecular weight of 50 kd appeared to be phosphorylated on tyrosine.

Key words: tyrosine kinase, phosphorylation, mitochondria, serum step-down, human fibroblast

A novel form of protein modification, the phosphorylation at tyrosine residues, has generated considerable interest regarding its potential involvement in the process of neoplastic transformation. Recently it has been indicated that at least nine known oncogenes encode proteins equipped with tyrosine-specific protein kinase activity [1]. Furthermore, it has been shown that phosphorylation on tyrosine represents one of the early events which follow the binding of cellular growth factors to their plasma membrane receptors [2], and it has been suggested that tyrosine phosphorylation of plasma membrane substrates may play a role in the regulation of cell proliferation [2,3].

Phosphorylation of protein tyrosine residues has been demonstrated in subcellular fractions other than plasma membrane, but very little information is available on the role of these kinases in the cell growth control [reviewed in 4]. In a number of normal and transformed animal cells we have recently described the association of a protein kinase activity, which phosphorylates tyrosine residues only in the presence

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of Mn^{2+} ions, with a postnuclear particulate cell fraction sedimenting at 12,000g [5]. In human diploid MRC5 fibroblasts we have further investigated the intracellular location and the endogenous substrates of this tyrosine kinase activity sedimenting at 12,000g. The MRC5 cells, whose proliferation rate is strictly dependent on cell density and serum concentration, offered the opportunity to investigate the relationship between changes of the cell proliferation rate and the extent of tyrosine residues' phosphorylation.

The results presented in this work indicate (1) the association of a tyrosine kinase activity with the mitochondrial outer membrane, (2) the modulation of this activity by the cell growth rate, and (3) the phosphorylation on tyrosine of a single protein band with an apparent molecular weight of 50 kd.

MATERIALS AND METHODS

Cells

MRC5 cells, human diploid fibroblasts derived from a female embryonic lung, were cultured and used between the 25th and 35th passage. MRC5 cells were grown as monolayer cultures in plastic Roux flasks at 37°C in air containing 5% CO_2 in Eagle basal medium with Earle's salts, containing 10% newborn calf serum (GIBCO, Paisley, Scotland), 50 U/ml penicillin, and 50 μg/ml streptomycin.

Purification and Characterization of Mitochondria

Mitochondria were purified from MRC5 cells according to the procedure described by Pedersen et al. [6] with slight modifications. The 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 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 200,000g for 30 min. The resulting pellet has been named 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 layered on the top of a 0.6–1.8 M continuous sucrose gradient and centrifuged at 120,000g for 90 min in an SW 27 Beckman-Spinco rotor. At the end of the run two bands, a small and a large one corresponding to a density range of 1.08–1.14 and 1.16–1.20 g/cm^3, respectively, were clearly visible in the gradient. In some experiments the large band corresponding to the mitochondrial fraction was collected, diluted in HSE solution, split in two aliquots, and centrifuged at 120,000g for 30 min. One of the pellets obtained was tested for marker enzymes after resuspension in the appropriate buffer; the other pellet was used for measuring the tyrosine kinase activity 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). All the operations were performed at 4°C. In other experiments to demonstrate the separation of mitochondrial outer membrane from the mitoplast and from contaminating organelles, the 0.6–1.8 M sucrose gradient was divided after the run into several fractions of increasing density.

To determine the grade of purity of the mitochondrial preparations or the separation of mitochondrial outer membrane from mitoplast the activities of marker enzymes were assayed on each fraction collected from the sucrose gradient. The activities of 5′-nucleotidase, acid phosphatase, glucose-6-phosphatase, NADH cytocrome C reductase, succinate dehydrogenase, and monoamino oxidase were determined [as described in 7–12, respectively]. Protein content of the PMPF and of the mitochondrial fractions was determined according to the method of Lowry et al. [13].

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). Reaction was started by adding 10 μl of the mitochondrial preparation (25 μg of proteins) 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 by 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 [14], 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, and the film was then developed.

Phosphoaminoacid Analysis

To determine phosphoaminoacid levels in the PMPF or in the mitochondrial 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 hr in tubes sealed under vacuum. 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 [5,15]. 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. When indicated monodimensional electrophoresis of the hydrolysates was performed at pH 3.5 for 90 min at 2.4 kV. Markers were detected by staining with ninhydrin, and to identify the ^{32}P-labeled phosphoaminoacids, an autoradiogram was made by exposing the electrophoretogram to Kodak X-omat X ray film for 1 day (see Fig. 1). The incorporation of ^{32}P was quantified by cutting out the appropriate paper pieces corresponding to the three phosphoaminoacid spots, placing them directly into scintillation fluid, and measuring the radioactivity by liquid scintillation spectrometry.

RESULTS

Association of Tyrosine Kinase Activity With Mitochondria

The purification grade of mitochondria was evaluated by testing marker enzymes for different subcellular fractions as obtained from resting and proliferating

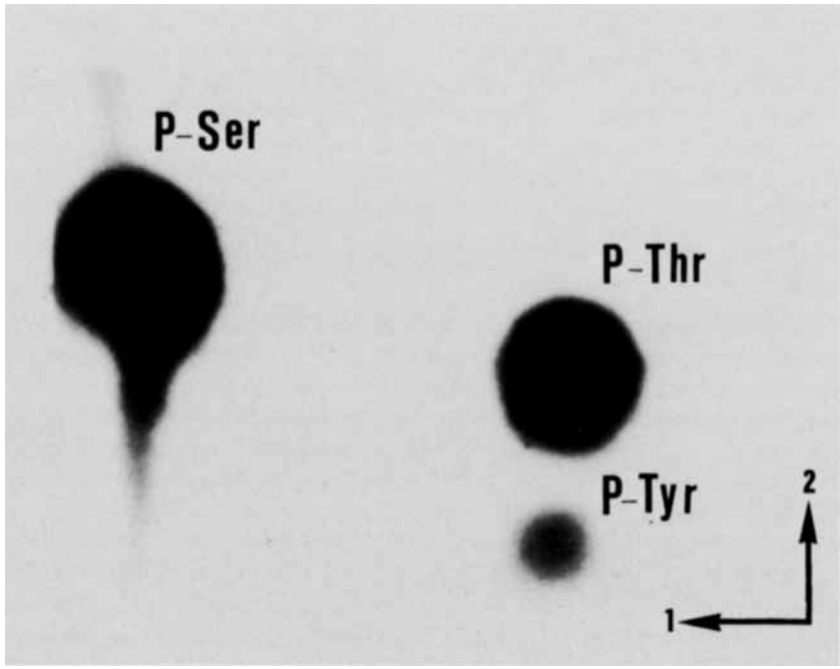

Fig. 1. Bidirectional electrophoresis of amino acids phosphorylated by protein kinase activities in mitochondria. Arrows on the autoradiogram indicate first and second directions of electrophoretic migrations from the origin.

MRC5 cells. The activities of 5′-nucleotidase (plasma membrane marker enzyme), glucose-6-phosphatase (microsomes), acid phosphatase (lysosomes), and succinate dehydrogenase (mitochondria) were estimated as described in the Materials and Methods section. Their distribution pattern in the purified mitochondrial fraction (M) and in the PMPF was analyzed by the de Duve plot [16]. As shown in Figure 2a, the de Duve plots of 5′-nucleotidase, acid phosphatase, and glucose-6-phosphatase activities resulted almost superimposable in quiescent or in proliferating cells. Furthermore, these enzyme activities appear to be enriched in the PMPF fraction and pratically absent in the M fraction. In this fraction, obtained either from quiescent or from proliferating cells, the relative specific activity of the mitochondrial marker enzyme succinate dehydrogenase (Fig. 2b) that resulted was about 20-fold enriched as compared to the activity present in the PMPF. It should also be noted that the relative specific activities of the marker enzymes tested were not affected by changes in the proliferation rate. Figure 2c shows that a tyrosine kinase activity was associated with the mitochondrial fraction and that its relative specific activity appeared to increase after serum step-down. From the data presented in this figure one can also note that, following serum step-down, an increase (from 12% to 18% of the total) of the mitochondrial proteins occurred.

In Table I, we can see the effect of Mn^{2+} ions on the tyrosine kinase activity present in the PMPF and in the mitochondria obtained from quiescent and proliferating cells. As shown, the presence of Mn^{2+} appeared to markedly increase the tyrosine kinase activity associated with the mitochondrial fraction but to marginally affect the activity of the tyrosine kinase present in the PMPF. Mn^{2+} dependence of the tyrosine

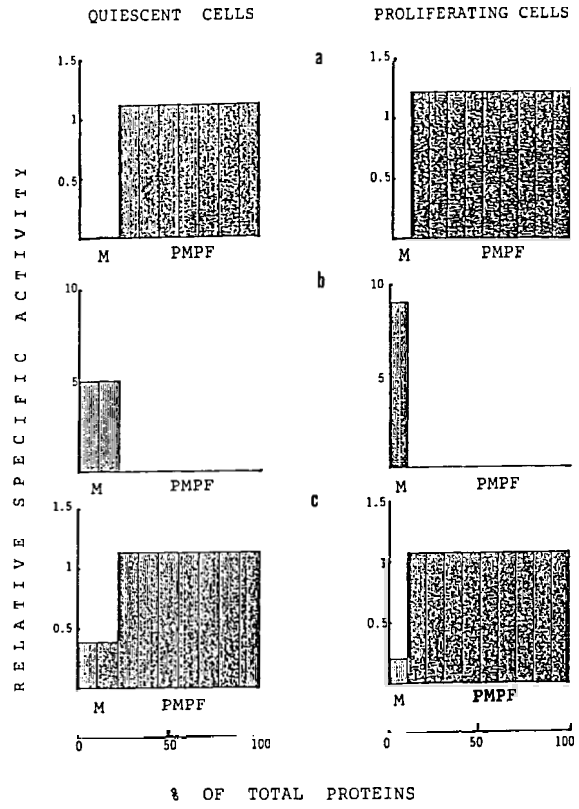

Fig. 2. The deDuve plot of the distribution patterns of tyrosine kinase and marker enzymes for subcellular organelles. Proliferating cells correspond to subconfluent cultures growing in the presence of 10% serum. Quiescent cells correspond to subconfluent cultures 48 hr after serum step-down from 10% to 0.5%. M, mitochondria; PMPF, postmitochondrial particulate fraction. a) 5′-nucleotidase, acid phosphatase, glucose-6-phosphatase; b) succinate dehydrogenase; c) phosphotyrosine kinase.

TABLE I. Effect on Mn^{2+} on Tyrosine Kinase Activity Present in the PMPF and in the Mitochondrial Fraction*

	Mitochondria		PMPF	
	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$
Quiescent cells	732	123	2,780	2,652
Proliferating cells	89	12	2,911	2,829

*Postmitochondrial particulate fraction (PMPF) and mitochondria of growing and quiescent cells were prepared as described in the Materials and Methods section. Tyrosine protein kinase reaction was carried out as previously described except that 10 mM $MnCl_2$ was present only where indicated. The assay was terminated by HCl addition. The tyrosine phosphorylation was evaluated by measuring the radioactivity of the phosphotyrosine spot after the bidirectional paper electrophoresis as described in the phosphoaminoacid analysis paragraph of the Materials and Methods. Values of tyrosine kinase activity were expressed as cpm of ^{32}P incorporated per μg of protein.

kinase activity occurred in mitochondrial fractions purified from both quiescent and growing cells.

Separation of Mitochondrial Outer Membrane From the Mitoplast

Our aim was then to separate outer membrane from the inner mitochondrial membrane and matrix and, concomitantly, to rule out the possibility that our mitochondrial preparation could be contaminated by other intracellular organelles. Therefore, the 0.6–1.8 M sucrose density gradient for the mitochondria purification was divided into several fractions of increasing density. On each of these fractions several marker enzymes, including monoamino oxidase (mitochondrial outer membrane specific enzyme), succinate dehydrogenase (mitochondrial inner membrane), acid phosphatase (lysosomes), 5′-nucleotidase (plasma membranes), glucose-6-phosphatase, and NADH-cytochrome C reductase (microsomes) were tested. As shown in Figure 3, the activity of 5′-nucleotidase and of both marker enzymes for microsomes sedimented at a density around 1.09–1.12 g/cm^3, whereas the activity of monoamino oxidase and succinate dehydrogenase sedimented at densities of 1.168 and 1.193 g/cm^3, respectively. It should also be noted that a small amount of acid phosphatase activity was detected in the fraction sedimenting at 1.248 g/cm^3. On the basis of the enzymatic and sedimentation analysis and of the data found in the literature [6,17] regarding submitochondrial fractions purification, it can confidently be stated that our mitochondrial fractions are free from other intracellular membrane contaminations and that the procedure used in this work clearly separates mitochondrial outer membrane from inner mitochondrial structures.

Separation of Tyrosine- From Serine- and Threonine-Kinase Activities

On each fraction corresponding to submitochondrial structures, as judged by the above-reported marker enzyme analysis, serine-, threonine-, and tyrosine-kinase activities have been tested. As shown in Figure 4, tyrosine kinase appeared to reach the maximum of its activity in the fractions that sedimented at a density of 1.17 g/cm^3, whereas serine and threonine kinases presented their maximum of activity in the fractions sedimenting at 1.19 g/cm^3.

Relationship Between Tyrosine-Kinase Activity and Growth Rate

Taking advantage of the serum sensitivity of the proliferation rate in this cell model, the phosphotyrosine content of mitochondria isolated from growing and resting cells, as obtained following alteration of the serum level, was investigated. As shown in Figure 5, the growth rate quotient (Q) of serum-fed cultures, estimated as previously described [18], continuously increased during the first 50 hr after cell seeding. Following serum step-down (from 10 to 0.5%), we recorded a rapid decrease of Q leading to very low values at about 70 hr after seeding. Low levels of tyrosine-kinase activity, as estimated by the in vitro phosphorylation of the mitochondrial fraction, were recorded during the first 50 hr after cell seeding when high Q values occurred. Following serum step-down and the consequent marked decrease of the growth rate, the phosphotyrosine content of mitochondria increased dramatically, reaching a maximum 20 hr after serum removal. It should be noted that following serum removal and consequent reduction of the cell growth rate the extent of serine and threonine phosphorylation was not affected (results not presented).

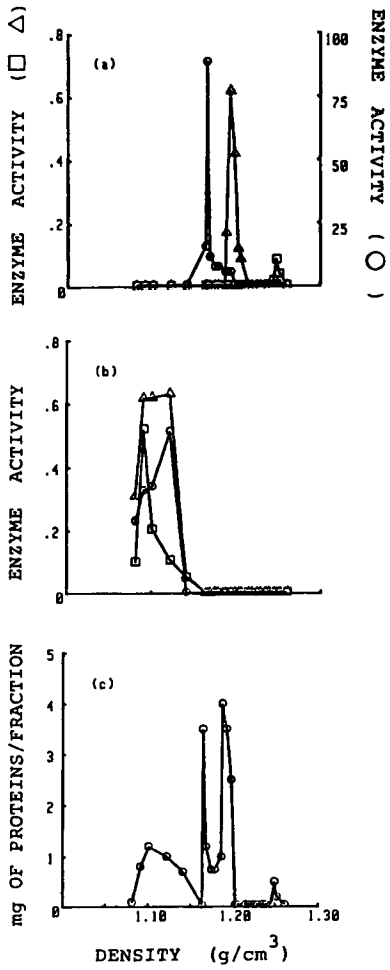

Fig. 3. Purification of submitochondrial particles on sucrose density gradient. After a 90-min run at 120,000g, the sucrose gradient was divided into several fractions of increasing density. On each fraction, marker enzyme activities (a, b) and protein content (c) were measured. The specific activities of succinate dehydrogenase (a, △), acid phosphatase (a, □), 5′-nucleotidase (b, □), glucose-6-phosphatase (b, △), and NADH cytocrome C reductase (b, ○) are expressed as μmols of substrate metabolized/mg of protein/min. The specific activity of monoamino oxidase (a, ○) is expressed as spectrophotometric units/mg of protein/min as previously reported [26].

Purified mitochondrial fractions enriched in their tyrosine kinase activity were incubated in the presence of [γ-^{32}P] ATP and $MnCl_2$ and the reaction products were separated by SDS-gel electrophoresis. As shown in Figure 6a, a single phosphorylated band of 50 kd was detectable in the autoradiogram. Moreover, the phosphorylation of this band occurred only in mitochondria isolated from quiescent cells. Phosphoaminoacid analysis of the 50-kd band extracted from the gel and hydrolyzed indicated tyrosine as the only phosphoaminoacid present (see Fig. 6b). A good correlation between the intensity of the 50-kd band and the level of tyrosine-kinase activity, as reported in Figure 5, was recorded in all the mitochondrial preparations tested.

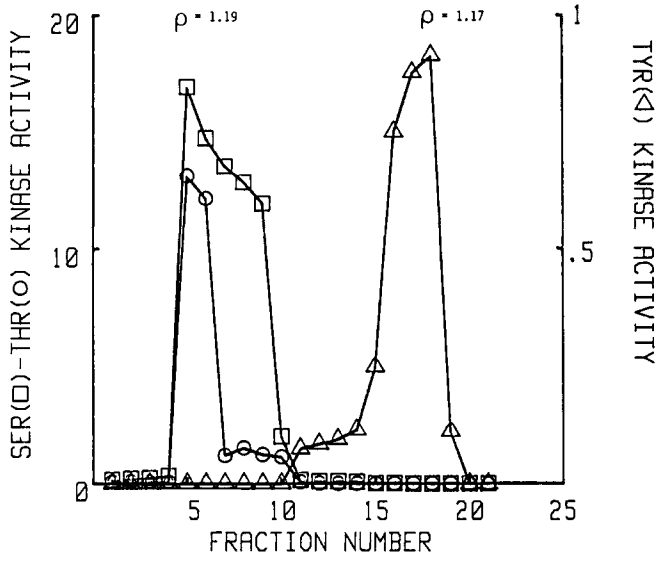

Fig. 4. Separation by sucrose gradient sedimentation of tyrosine-, serine-, and threonine kinase activities associated with the mitochondrial fraction. After a 90-min run at 120,000g, the large band corresponding to a density range of 1.16–1.20g/cm^3 was eluted into several fractions. On each of these fractions protein kinase activities were assayed as reported in the Materials and Methods section. Values of kinase activities were expressed as cpm of $^{32}P \times 10^{-3}$ incorporated per μg of protein.

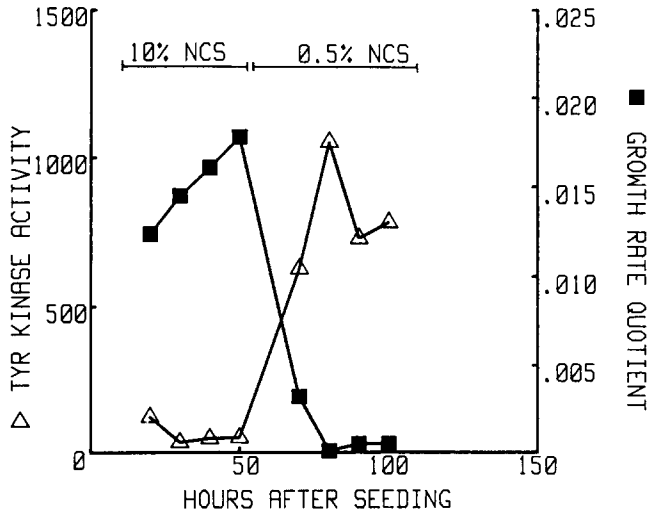

Fig. 5. Effect of serum step-down on proliferation rate and tyrosine kinase activity in MRC5 cells. Subconfluent growing cells were deprived of serum (from 10% to 0.5%) 50 hr after seeding. Tyrosine kinase activity, expressed as cpm of $^{32}P \times 10^{-3}$ incorporated per μg of protein, was evaluated at the indicated times as described in the Materials and Methods section. Proliferation rate was estimated by the growth rate quotient calculated as previously described [18] at the indicated times.

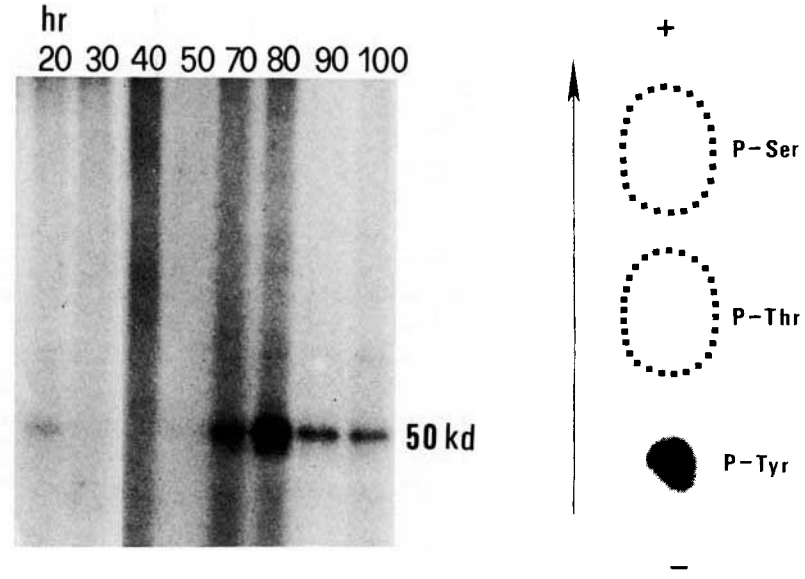

Fig. 6. PAGE analysis of endogenous protein substrates phosphorylated in vitro. In a, mitochondrial fractions sedimenting at a density of 1.17 g/cm^3 and enriched in tyrosine kinase activity were purified from growing cultures at the indicated times before and after serum step-down occurring 50 hr after seeding. These fractions were assayed for protein kinase activity and the reaction products were analyzed by PAGE under the standard conditions described in the Materials and Methods section. The molecular weights of the phosphorylated substrates were calculated by comparing their relative mobilities with those of comigrated protein standards. In b, the 50-kd band cut from the gel was hydrolyzed and subjected to monodimensional electrophoresis as described in the Materials and Methods.

DISCUSSION

The data presented in this work indicate that a tyrosine kinase activity, as determined by an in vitro phosphorylation reaction, is associated with mitochondria purified from diploid human fibroblasts. Wong and Goldberg [19] have previously reported an association of tyrosine kinase activity with subcellular components including mitochondria obtained from rat liver. They have also reported that the extent of mitochondrial tyrosine phosphorylation corresponds to a small aliquot (2.2%) of the total cellular tyrosine kinase activity. A contamination of mitochondrial fraction by other subcellular organelles could explain the association of tyrosine kinase with this fraction. For this reason, we previously analyzed the purification degree of the mitochondrial fraction by looking at specific enzyme distribution patterns and by using sedimentation density criteria. By marker enzyme analysis our preparation has clearly been identified as highly purified mitochondria. This conclusion has been supported by the result of the gradient sedimentation analysis, indicating for the mitochondrial fraction density values of 1.17–1.19 g/cm^3 that confirm those reported in the literature [6,17,20].

The unexpected result on the separation by gradient sedimentation analysis of outer membrane from inner membrane and matrix as judged by specific marker enzyme analysis indicated an increased fragility of intracellular organelles when separated from cells lysed by a hyposmotic shock. The possible disruption of the mitochondrial structure when subjected to isopycnic centrifugation at high speed has

previously been reported [21,22]. Both of these treatments could well have induced selective removal of the outer from the inner membrane and the mitochondrial matrix during our purification procedure. The result on the phosphoaminoacid analysis performed on the sucrose gradient fractions indicated that tyrosine kinase activity cosedimented with monoamino oxidase activity, whereas threonine- and serine kinase activities cosedimented with succinate dehydrogenase activities. The above indicated that tyrosine kinase is associated with the mitochondrial outer membrane.

The marked dependence of mitochondrial tyrosine kinase activity on Mn^{2+} ions in vitro can be explained on the grounds that in our assay a low level of ATP has been used, and it has been reported that the affinity of some tyrosine kinases for Mn^{2+}-ATP is higher than that for Mg^{2+}-ATP [4]. Furthermore, Mn^{2+} appears to inhibit particulate tyrosine-specific phosphatase activities [23]. That this should be the case is also suggested by the evidence that mitochondria from MRC5 cells as well other cell types are endowed with tyrosine-specific phosphatase activity: indeed, our unpublished observations have shown that tyrosine phosphorylation of mitochondria performed in vivo was detectable only in the presence of a specific inhibitor of tyrosine phosphatase such as vanadate [24].

The striking change in the extent of tyrosine phosphorylation of the 50-kd phosphoprotein associated with the external mitochondrial membranes that follows serum step-down might suggest a role for the mitochondrial tyrosine kinase in the regulation of cell growth. Moreover, the well-known reduction of mitochondria content in growing tissues [21] should enlarge the difference in tyrosine phosphorylation between mitochondria of quiescent and growing fibroblasts. At present, we do not have knowledge of a plausible role of specific mitochondrial proteins in the control of cell proliferation. However, recently Piga et al. [25] have shown that the tyrosine protein kinase activity associated with a particulate fraction (including mitochondria) of human lymphocytes declines when the cells are activated by PHA addition. Their results and the observed reduction of tyrosine kinase activity associated with mitochondria obtained from proliferating human fibroblasts support the hypothesis that in subcellular compartments other than plasma membrane, marked decreases of tyrosine kinase activity occur during the proliferative commitment.

ACKNOWLEDGMENTS

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REFERENCES

1. Sefton BM: Trends in Genetics 1:306, 1985.
2. Heldin CH, Westermark B: Cell 37:9, 1984.
3. Hunter T, Gould KL, Cooper JA: Biochem Soc Trans 12:757, 1984.
4. Swarup G, Dasgupta JD, Garbers DL: Adv Enzyme Regul 22:267, 1984.
5. Montagnier L, Chamaret S, Dauguet C: C R Acad Sci 295T:375, 1982.
6. Pedersen PL, Greenawalt JW, Reynafarje B, Hullihen J, Decker DL, Soper JW, Bustamente E: Methods Cell Biol 20:411, 1979.
7. Michell RH, Hawthorne JN: Biochem Biophys Res Commun 21:333, 1965.

8. Bergmeyer HY: In "Methods in Enzymatic Analysis." New York: Academic Press, 1970, p 613.
9. Hubscher G, West GR: Nature 206:799, 1965.
10. Fleischer B: Methods Enzymol 31:180, 1974.
11. Earl DCN, Korner A: Biochem J 94:721, 1965.
12. Tabor CW, Tabor H, Rosenthal SM: J Biol Chem 208:645, 1954.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RY: J Biol Chem 193:265, 1951.
14. Laemmli UK: Nature 227:680, 1970.
15. Ushiro H, Cohen S: J Biol Chem 255:8363, 1980.
16. de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F: Biochem J 60:604, 1955.
17. Reid E, Williamson R: In Fleischer S, Packer L: (eds): "Methods in Enzymology," 31:713, 1974.
18. Piedimonte G, Borghetti AF, Guidotti GG: Cancer Res 42:4690, 1982.
19. Wong TW, Goldberg AR: Proc Natl Acad Sci USA 80:2529, 1983.
20. Maunsbach AB: Methods Enzymol 31:330, 1974.
21. Pedersen PL: Prog Exp Tumor Res 22:190, 1978.
22. Wattiaux R, Wattiaux-De Coninck S: Biochem Biophys Res Commun 40:1185, 1970.
23. Casnellie JE, Harrison ML, Pike LJ, Hellstrom KE, Krebs EG: Proc Natl Acad Sci USA 79:282, 1982.
24. Leis JF, Kaplan NO: Proc Natl Acad Sci USA 79:6507, 1982.
25. Piga A, Wickremasinghe RG, Taheri MR, Yaxley JC, Hoffbrand AV: Exp. Cell Res. 159:103, 1985.
26. Yasumbo KT, Smith RH: Methods Enzymol. 17:698, 1971.