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PHOSPHOPROTEIN ASSOCIATED WITH ACTIVATION OF THE SRC GENE PRODUCT IN MYOGENIC CELLS

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SUMMARY

Chicken mononucleated myoblasts were infected with a temperature sensitive mutant of Rous sarcoma virus (tsNY68) and allowed to develop into myotubes by incubating at 41°C (nonpermissive temperature for the src gene product). Activation of the src gene product by temperature shiftdown to 36°C (permissive temperature) in these myotubes was found to phosphorylate a 36,000 dalton cellular protein which is similar to the one reported by Radke and Martin (1) recently. Phosphorylation of this 36,000 dalton protein was observed within 3 hr after temperature shift. No appreciable phosphorylation of this protein was observed in control myotubes kept at 41°C nor in the uninfected myotubes. These results suggested that this 36,000 dalton protein may be a possible target of the src gene product ($pp60^{src}$) of Rous sarcoma virus for its cellular transformation in myotubes as well as in fibroblasts.

INTRODUCTION

In our preceeding communication, we reported that mononucleated myoblasts can be infected with a mutant of Rous sarcoma virus (RSV) which has a temperature sensitive lesion in the src gene (2). When the temperature of such a myoblast culture was maintained at the nonpermissive temperature (41°C) normal development took place and cells fused, forming myotubes. Upon temperature shift down of the culture, to the permissive temperature (36°C), vacuoles were observed with concomitant induction of DNA synthesis in terminally differentiated myotubes. Such an induction of DNA synthesis in myotubes is unique to this system and mechanism through which activation of the src gene induces such DNA synthesis may be studied in these relatively quiescent, nongrowing cells. It has been shown recently that RSV src gene codes for a protein having a molecular weight of 60,000 daltons which can phosphorylate itself as well as other

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proteins (3). It has been proposed that the src gene product ($pp60^{src}$) therefore phosphorylates target protein(s) of the host cell which in turn will manifest various phenotypic expressions of transformed cells such as induction of DNA synthesis. We searched for the protein which can be phosphorylated under the condition which induces DNA synthesis and vacuole formation in myotubes. If such protein(s) exist, phosphorylation of this protein(s) should be observed upon activation of the src gene within a relatively short period. The search for such a target protein in relatively quiescent cells such as myotubes (4) should be easier than in the actively growing fibroblasts where phosphorylation of various proteins takes place actively. We have found a protein with approximately a 36,000 dalton molecular weight that is rapidly phosphorylated upon temperature shift down to 36°C of myotubes which had been infected with tsNY68 (5) at the stage of mononucleated myoblasts and allowed to develop into multinucleated myotubes at 41°C. From the behavior of this protein in the two dimensional gel electrophoresis, it appears that this protein is equivalent to the one which has been reported in RSV transformed fibroblasts suggesting that the target protein may be identical, regardless of the origin of the tissue.

MATERIALS AND METHODS

Myogenic cell culture. Chicken myogenic cells were prepared by dissecting breast muscle from avian leukosis virus free 11 day old chick embryos. (SPAFAS Lancaster, PA). The mononucleated muscle cells were seeded at a concentration of 7×10^5 cells per 35 mm x 10 mm gelatin coated plastic dish and cultured in 8:1:1 medium (100 ml of Eagle's minimum essential medium, 12.5 ml of horse serum, 12.5 ml of chicken embryo extract, 1.5 ml of penicillin, streptomycin mixture (penicillin 10^4 units/ml, streptomycin 10 mg/ml), 1.5 ml of 200 mM glutamine, 1.5 ml of Fungizone (250mg/ml)). Cells were infected with tsNY68 at the time of plating (approximately 1 focus forming unit per cell) and maintained at 41°C. Two days after plating, cytosine arabinonucleoside (araC), an inhibitor of DNA synthesis, was given at a final concentration of 1 µg/ml to eliminate possible contamination of fibroblasts.

Labeling cell and two dimensional gel electrophoresis. On day 3 or 4 after the myotube formation was completed, the temperature of the culture was lowered to 36°C and cells were labeled with [^{32}P]phosphoric acid (NEN carrier free) with a concentration of 1 mCi/ml (ICi = 3.7×10^{10} bequerels) for 3 hr in MEM (minimum essential medium) supplemented with 0.1% bovine serum albumin. The culture was then washed 3 times with cold MEM and lysed in 0.1 ml of lysis buffer (9.5 M Urea, 2% Nonidet P-40, 2% Amphorine) and subjected to two dimensional gel electrophoresis. Non equilibrium pH gradient electrophoresis (NEPHGE)

was in the horizontal dimension (pH 3-10) and electrophoresis was conducted at 400V/11.7 cm for 6 hrs at room temperature. Sodium dodecylsulfate (SDS) - polyacrylamide gel electrophoresis (10%) was performed with 20 mA constant current in the vertical dimension. About 5×10^5 c.p.m. was subjected to each gel. After 2nd dimension electrophoresis gels were fixed with 50% TCA (trichloroacetic acid) and dried. Dried gel was exposed to Kodak XR-5 film with intensive screen for 75 hr at -70°C.

RESULTS AND DISCUSSION

Chicken myoblasts were isolated from 11 day old leukosis virus-free chicken embryo (SPAFAS) and were infected with tsNY68 which was kindly provided by H. Hanafusa of Rockefeller University. Cultures were kept at 41°C and allowed to form multinucleated

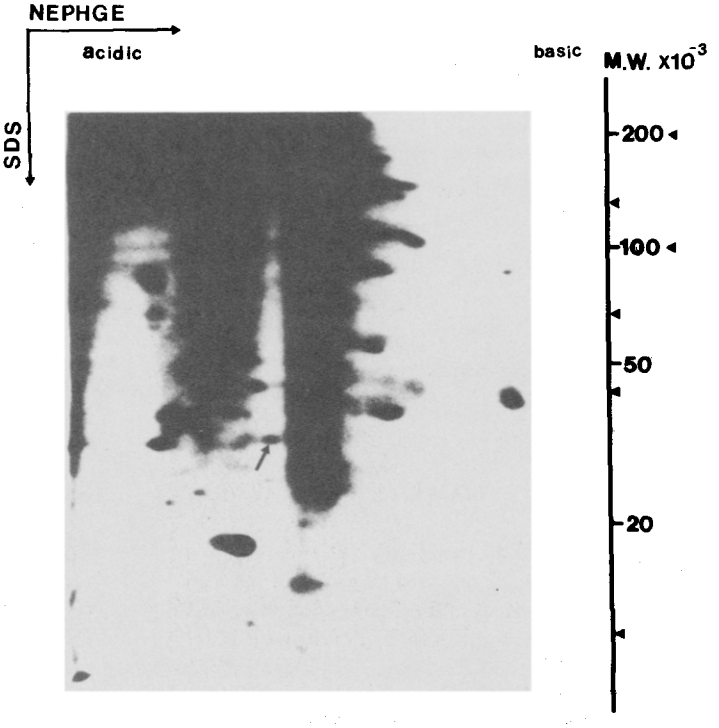

Figure 1: ^{32}P labelled 36K protein (pp36K) observed in tsNY68 infected chicken myotube upon activation of the src gene product.

Chicken myogenic cells were prepared and labeled with [^{32}P] phosphate as described in Materials and Methods and 2-dimensional gel electrophoresis was performed. For the molecular weight determination, the following proteins were subjected to 2-dimentional gel electrophoresis: Myosin (MW, 200×10^3), β-galactosidase (MW, 130×10^3), phosphorylase a (MW, 100×10^3), bovine serum albumin (MW, 68×10^3), ovalbumin (MW, 43×10^3), and cytochrome c (MW, 11.7×10^3). The positions of the marker proteins are shown in this Figure (◀). The arrow indicates the 36K spot which appears upon activation of the src gene.

myotubes. After the formation of myotubes under these conditions the temperature of these cultures was shifted to 36°C and [^{32}P] phosphate was given for 3 hr., then myotubes were harvested and subjected to 2- dimensional gel electrophoresis as described by O'Farrell et al., (6). As can bee seen in fig. 1 the radioactive spot, shown by the arrow, which has 36,000 dalton molecular weight, appeared distinctly upon activation of the src gene product. This [^{32}P] labeled 36,000 dalton molecular weight spot was examined for its sensitivity to pronase or nuclease digestion. It was found that about 80% of its radioactivity was eluted from the gel after treatment with pronase (50µg/ml) for 15 hrs at 37°C while less than 10% was eluted by DNase or RNase (50µg/ml) under identical conditions. In a separate experiment, myotubes were labeled with [^{32}P] phosphate and [^{35}S]-methionine simultaneously. The 36,000 dalton molecular weight spot could be observed either by [^{35}S]-methionine radioactivity or by [^{32}P] radioactivity indicating that the 36,000 dalton spot is indeed a phosphoprotein (data not shown). We therefore tentatively conclude that the 36,000 dalton radioactive spot is a phosphoprotein and named this phosphorylated 36,000 dalton protein, pp36K. It is clear from Fig. 2 (a) that an extract of non infected myotube culture did not contain appreciable amount of pp36K under similar conditions. Only a trace of radioactivity may be observed occasionally but most of the time no significant radioactivity could be observed in this spot. Fig. 2 (b) shows the pp36K spot under identical enlargement as Fig. 2 (a) for comparison. As shown in table 1 the phosphorylation of this 36K protein can take place only by the activation of the src gene product and within 3 hr after temperature shift up from 36°C to 41°C of the culture, pp36K protein disappears. The low level radioactivity detected at this spot may simply represent background radioactivity. On the other hand it is possible that low level pp36K may exist in normal cells because a low level pp60^{src} exists even in normal cells (7). A slight radioactivity occasionally (1 out of 5 experiments in total) noticed at the spot of pp36K in normal myotubes may represent such low level of pp36K in normal cells.

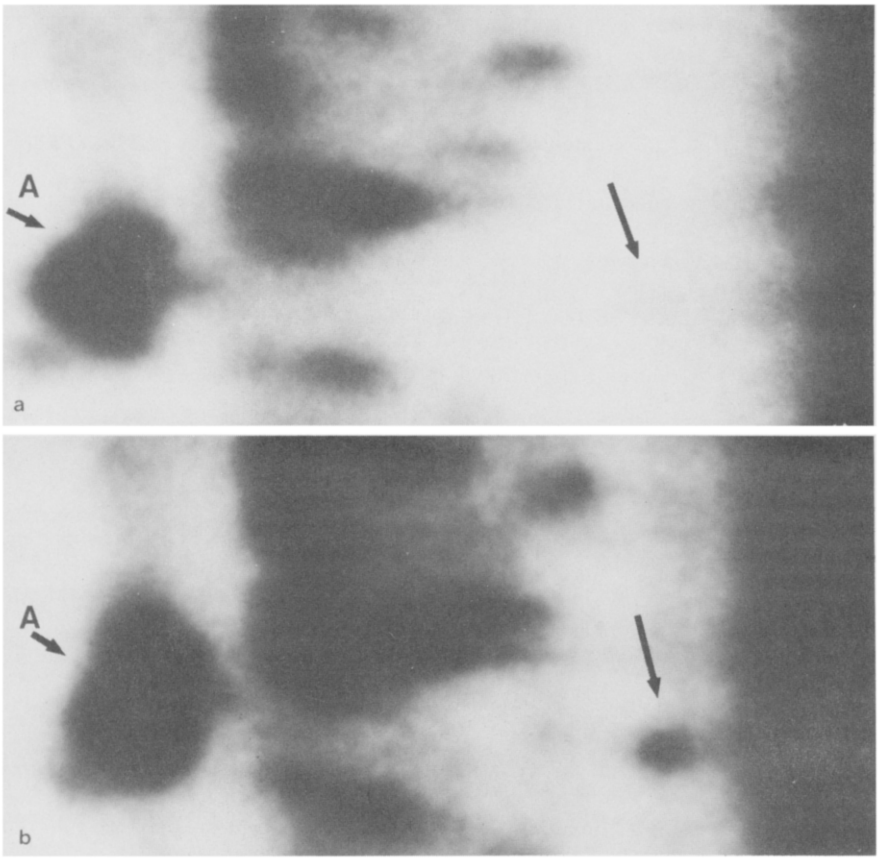

Figure 2: Absence of pp36K in normal myotube culture.

Normal myotube culture was prepared and cultured at 41°C for 3 days under the same conditions as Fig. 1. Upon the temperature shift of these cultures to 36°C, cells were labelled with [^{32}P] - phosphate for 3 hr and subjected to 2-dimensional gel electrophoresis as in Fig. 1. A portion of the autoradiogram is enlarged.
(a) Normal myotube culture. The arrow indicates the position of pp36K, showing the absence of this phosphoprotein.
(b) tsNY68 infected myotube. The experiment was carried out under the same conditions as Fig. 1. The arrow indicates pp36K.
Spot A was observed regardless of temperature or virus infection and used as a reference radioactivity spot in calculating relative radioactivity of pp36K as shown in Table 1.

We do not have, at the present moment, evidence that pp36K is the direct target protein of the pp60^{src} for cellular transformation. Considering the rapidity of appearance and disappearance of pp36K as shown in table 1, pp36K could be one of the target proteins of the src protein. Cell transformation markers, such as disappearance of fibronectin (8), change of sugar transport (9), activation of plasminogen activator (10) etc., are expressed slower

TABLE 1 Disappearance of pp36K upon temperature shift up to 41°C

		RADIOACTIVITY (cpm)		
Virus Infection	Temperature	pp36K	Spot A	Ratio $\frac{(pp36K)}{(Spot\ A)}$
Noninfected	41 (a)	1.5	66.3	0.023
	41→36 (b)	1.3	47.3	0.026
Infected	41 (a)	1.5	66.5	0.023
	41→36 (b)	27.5*	77.6*	0.354
	41→36→41 (c)	1.5	74.3	0.020

Each sample was subjected to 2-dimentional gel electrophoresis as described in Fig. 1. Following the autoradiogram, pp36K was cut out from the gel, its radioactivity counted, and was normalized by the count of spot A (Shown in Fig. 2) used as an internal standard marker, and the ratio of cpm of pp36K to that of spot A is calculated.

(a) Myotubes were labeled with [^{32}P] phosphate as in Fig. 1 for 3 hr at 41°C.
(b) Immediately after temperature was shifted down to 36°C, pulse of [^{32}P] phosphate was given for 3 hr as in Fig. 1.
(c) Culture was shifted down to 36°C for 3 hr and raised to 41°C again with simultaneous pulse of [^{32}P] phosphate for 3 hr at 41°C.
* Average of 20 gels
Other values were average of 4 gels

than the appearance of pp36K and phosphorylation of 36K protein may lead to various phenotype expression of the src gene. However, the possibility exists that pp36K protein, since the molecular weight of pp36K is smaller than 60,000 (MW of the src protein), may represent a degradation product of the src gene. In fact, Sefton et al., (11) reported that degraded src protein (50K) might exist in transformed fibroblasts. However, our preliminary studies using anti pp60^{src} and [^{32}P] labeled fibroblast indicated no pp36K protein precipitated by this antibody (data not shown). Further studies with myotubes are necessary to establish this point. Since only p19 is a phosphorylated RSV viral structural protein (12), the possibility that pp36K represents viral protein other than the src protein appears to be small. Temperature dependent phosphorylation of 36K protein in RSVtsLA29 infected fibroblasts has recently been reported (1). The position, in the two dimensional gel electrophoresis analysis strongly suggest that these proteins are identical. Further experiments should be carried out to establish the identity of these two proteins.

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