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Biochemistry

Phosphorylation of ribosomal protein S6 in avian sarcoma virus-transformed chicken embryo fibroblasts

(serum starvation/growth factors/temperature-sensitive mutant)

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ABSTRACT **Protein phosphorylation was examined in whole cell extracts from normal and avian sarcoma virus-transformed chicken embryo fibroblasts. The addition of serum or epidermal growth factor to serum-starved normal cells resulted in increased ^{32}P labeling of a M_r 30,000 protein. In extracts from cells transformed by a temperature-sensitive mutant of Schmidt–Ruppin virus, subgroup A, and grown at the permissive temperature, the protein was phosphorylated regardless of serum starvation. This M_r 30,000 protein was shown to be ribosomal protein S6, and the effects of avian sarcoma virus transformation on S6 phosphorylation were further investigated. The ability to phosphorylate S6 in the absence of serum was found to be temperature sensitive when S6 preparations from the temperature-sensitive mutant-infected cells incubated at permissive and nonpermissive temperatures were compared. Cells transformed by the parent virus (Schmidt–Ruppin, subgroup A) maintained the ability to phosphorylate S6 in the absence of serum when incubated at either temperature. Phosphoserine was the only phospho-amino acid detected in acid hydrolysates from phosphorylated S6 preparations.**

Recent investigations have emphasized the role of protein phosphorylation in growth regulation in normal cells and in the establishment and maintenance of the malignant state in virally transformed cells (1–3). Mitogenic agents such as epidermal growth factor (4), insulin (5), and insulin-like growth factor (6) have been shown to enhance phosphorylation of certain cellular proteins. In particular, all these growth-promoting peptides have been shown to stimulate the phosphorylation of ribosomal protein S6 when added to quiescent cell cultures (7), and it has been suggested that phosphorylation of S6 may be required for the transition of serum-deprived cells into the G_1 phase of the cell cycle (8). S6 has been shown to be the primary poly(U) binding protein of the 40S ribosomal subunit (9), and it has been postulated that phosphorylation of S6 alters the affinity of the ribosome for certain classes of mRNAs (7).

Most transformed cell lines show a reduced requirement for serum and are able to grow in the absence of at least one normally required growth factor (10). If, indeed, growth factor-induced protein phosphorylation is important in the initiation of growth, these transformed cells have acquired the ability to perform such phosphorylation reactions independently of normally needed serum factors.

In this report, the phosphorylation state of ribosomal protein S6 has been determined in avian sarcoma virus (ASV)-infected cells under various conditions of serum deprivation.

MATERIALS AND METHODS

Cell Cultures. Subconfluent secondary cultures were used in all experiments involving chicken embryo fibroblasts. Primary cultures were prepared from 10- to 11-day-old embryos as described by Vogt (11). Secondary cultures were seeded in Scherer's maintenance medium/10% tryptose phosphate/5% calf serum containing beef embryo extract at 1.25–2.50 × 10^6 cells per 100-mm dish. ASV-transformed rat cell line RR 1022 (obtained from A. Goldberg, Rockefeller University) was seeded at 1 × 10^6 cells per dish in Dulbecco's modified Eagle's medium/10% calf serum. After 2 days incubation at 36°C, the cells were used for ^{32}P-labeling experiments. Transformed chicken embryo fibroblasts were prepared by infecting primary cultures with the Schmidt–Ruppin strain, subgroup A, of ASV (SR-A) or with a temperature-sensitive mutant derived from this strain (ts 68) (both viruses kindly provided by H. Hanafusa, Rockefeller University).

$NaDodSO_4$/Polyacrylamide Electrophoresis of Whole Cell Lysates. Two-day-old subconfluent cultures were drained and washed twice with 5 ml of Scherer's medium lacking phosphate, calf serum, beef embryo extract, penicillin, streptomycin, and phenol red and then incubated in 5 ml per dish of the same medium containing 100–200 μCi of carrier-free ^{32}P (1 Ci = 3.7 × 10^{10} becquerels; Amersham) per dish. After 6 hr, some samples received 5% calf serum or epidermal growth factor at 500 ng/ml (Collaborative Research, Waltham, MA) for 15 min. Cells were then washed twice with 5 ml of TMK buffer (5 mM Tris·HCl/10 mM $MgCl_2$/80 mM KCl, pH 7.4) and lysed with 0.3 ml of 2% $NaDodSO_4$/10% sucrose/5% mercaptoethanol. Lysates were heated at 100°C for 5 min and run on 5–16% $NaDodSO_4$/polyacrylamide gels as described by Laemmli (12).

Preparation of Ribosomal Pellets and Polyacrylamide Gel Electrophoresis of Ribosomal Proteins. Ribosomes were prepared by a modification of the method of Haselbacher *et al.* (6). Cells were grown and labeled with ^{32}P as described above. After the 6-hr labeling period, the monolayers were washed twice with 5 ml of TMK buffer and lysed with 2 ml per dish of cold TMK buffer/1% sodium deoxycholate/1% Triton X-100/10 mM KH_2PO_4/2 mM *p*-aminobenzamidine dihydrochloride/2 mM l-*p*-bromotetramisole oxalate, pH 7.4. Extracts were centrifuged at 30,000 × *g* for 10 min, and the supernatants were layered over a cushion of 1.5 M sucrose in TMK buffer and spun at 100,000 × *g* for 2 hr. Pellets were washed and resuspended in TMK buffer. RNA was precipitated with 100 mM $MgCl_2$/67% acetic acid, and the precipitate was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was precipitated with 5 vol of cold acetone. Two-dimensional gel electrophoresis was performed using the method of Ramjoue and Gordon (13), except that the first dimension was run on 1-mm-thick slab gels (200 μg of protein per track) at 10 mA for 1 hr and 55 mA for 16 hr rather than in tubes. The first-dimension

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Abbreviations: ASV, avian sarcoma virus; SR-A, Schmidt–Ruppin strain, subgroup A of ASV; ts 68, temperature-sensitive mutant derived from SR-A.

gel was dried and exposed, and tracks were cut out for the second-dimension gels, which were run at 10 mA for 1 hr and 50 mA for 8–10 hr. For direct quantification of ^{32}P incorporation, labeled bands were cut from the first-dimension gels and assayed in Hydrofluor (National Diagnostics) in a Packard Tri-Carb liquid scintillation counter.

Phospho-Amino Acid Analysis. Identification of phosphorylated amino acids from partially hydrolyzed protein was done by one-dimensional high-voltage electrophoresis as described by Hunter and Sefton (14). ^{32}P-Labeled S6 protein bands cut from dried first-dimension urea gels were washed and rehydrated in 50 mM $NaHCO_3$/0.1% $NaDodSO_4$. Gel bands were crushed with a glass pestle and shaken for 18 hr at 37°C. The polyacrylamide fragments were removed by centrifugation, 100 μg of ovalbumin was added to the eluted protein in the supernatant, and protein was precipitated with 20% cold trichloroacetic acid. The pellet was washed with ethanol at −10°C, then with ice-cold ethanol/ether (1:1), dried, and dissolved in 6 M HCl. This solution was placed in a tube, which was sealed at reduced pressure, and heated at 110°C for 2 hr. The hydrolysate was lyophilized and subjected to high-voltage electrophoresis at pH 3.5 in glacial acetic acid/pyridine/H_2O, 50:5:945 (vol/vol).

Miscellaneous Measurements. ATP was measured by using a luciferin-luciferase assay (15), and protein was determined by using either the method of Lowry *et al.* (16) or that of Bradford (17).

RESULTS

^{32}P-Labeling in Whole Cells. The banding patterns of the ^{32}P-labeled macromolecules in whole cell lysates of ^{32}P-labeled cells are shown in Fig. 1. Serum-starved subconfluent normal cells display similar labeling compared with identically treated cells that had been incubated with 5% calf serum for 15 min before $NaDodSO_4$ solubilization. The only obvious difference is the presence of a heavily labeled M_r 30,000 band in the serum-treated cells. The same experiment was performed using ts 68-infected cells incubated at the permissive temperature (36°C). With these cells, phosphorylation of the M_r 30,000 band was found to be independent of serum addition; similar heavy labeling occurred in the presence or absence of serum. Presumably, this M_r 30,000 band represented a phosphorylated protein because it was insensitive to ribonuclease treatment and sensitive to protease.

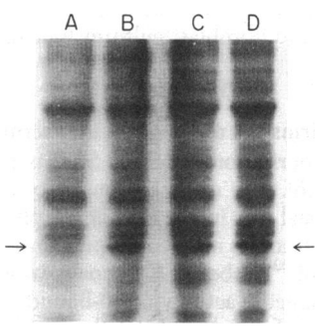

FIG. 1. Autoradiogram of $NaDodSO_4$ extracts of ^{32}P-labeled whole cells subjected to electrophoresis on $NaDodSO_4$/polyacrylamide gels. Lanes: A, serum-starved normal cells; B, duplicate cultures incubated with 5% calf serum for 15 min before extraction; C, serum-starved ts 68-transformed cells incubated at 36°C; D, ts 68-transformed cells incubated with 5% serum at 36°C. Arrows indicate migration position of the M_r 30,000 protein, calculated from the mobilities of ovalbumin, DNase I, and soybean trypsin inhibitor. Each lane contained 150 μg of protein. Gels were exposed for 24 hr.

Identification of the M_r 30,000 Protein. It has previously been shown that serum and certain growth factors and hormones enhance the phosphorylation of ribosomal protein S6, a protein that has an estimated M_r of 28,000–34,000 (7). Ribosomes from *in vivo* ^{32}P-labeled normal and ASV-infected cells were partially purified, and phosphorylation of basic ribosomal proteins was examined by using two-dimensional urea gel electrophoresis. The S6 protein in these preparations was identified by comparison of the Coomassie blue staining patterns with gel patterns described previously (8), in particular, by substantiating the decrease in mobility toward the cathode as the protein is progressively phosphorylated [S6 exists in at least five phosphorylation states (8)]. As shown in Fig. 2, the S6 protein from serum-starved normal cells was predominantly in the less phosphorylated cathodal position and lined up diagonally with proteins S2 and S4. However, when serum was added, the position of the S6 protein shifted toward the anode such that the predominantly stained portion was to the anodal side of protein S4 (Fig. 2*C*). In the autoradiograms from these gels, very little label was incorporated into the heavily stained spot from serum-starved cells while the major spot from serum incubated cells was strongly labeled. These observations are largely in agreement with those of Haselbacher *et al.* (6). Gels of basic ribosomal proteins from ts 68-infected cells are shown in Figs. 3 and 4. When the cells were serum-starved at the permissive temperature (36°C), S6 was located in the heavily phosphorylated anodal position, regardless of the presence or absence of serum (Fig. 3). In contrast, when cells were ^{32}P-labeled at the nonpermissive temperature (42°C) in the absence of serum, S6 phosphorylation was minimal (Fig. 4), with the Coomassie blue staining spot positioned as in serum-starved normal cells. Addition of serum to these cells stimulated phosphorylation of S6 and caused the anodal shift to occur (Fig. 4*C*). When similar experiments were done using cells transformed by the wild-type virus (SR-A), S6 remained phosphorylated in the absence of

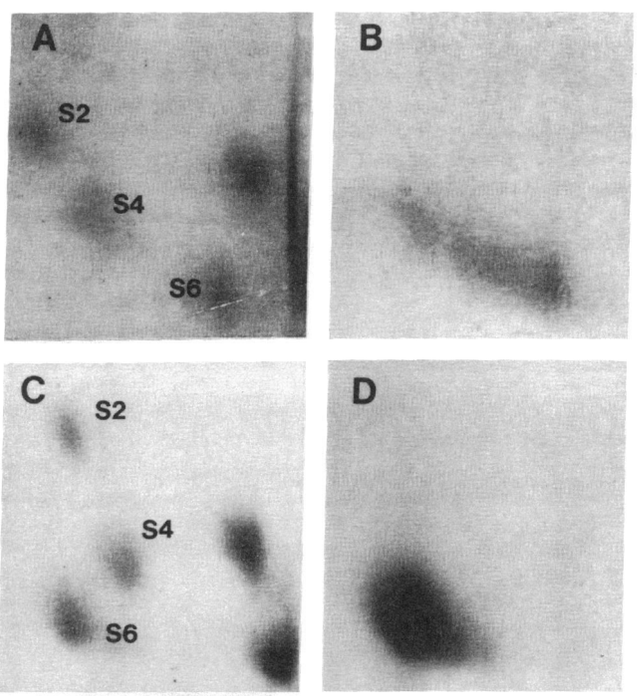

FIG. 2. Coomassie blue staining patterns (*A* and *C*) of two-dimensional urea gels of ^{32}P-labeled basic proteins and autoradiograms (*B* and *D*) made from these gels. (*A* and *B*) Proteins from serum-starved normal cells. (*C* and *D*) Proteins from cells incubated for 15 min with 5% calf serum.

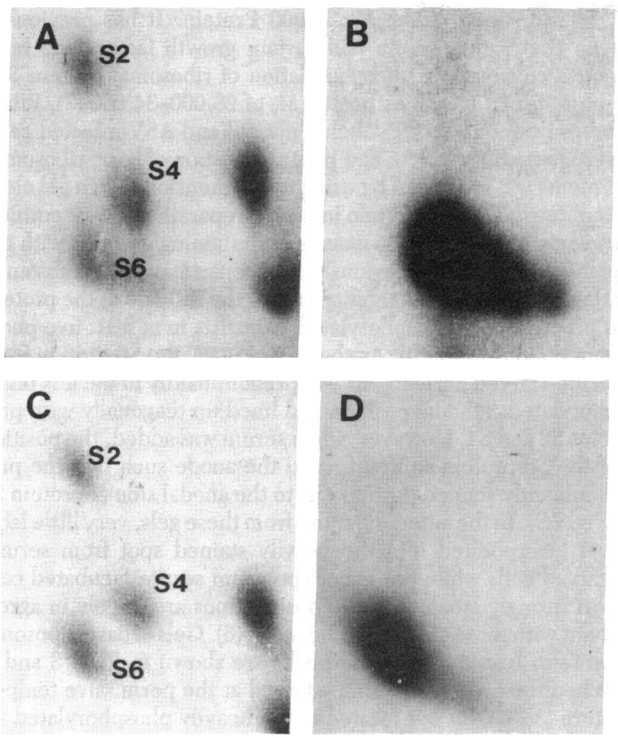

FIG. 3. Coomassie blue staining patterns (*A* and *C*) and autoradiograms (*B* and *D*) of two-dimensional urea gels of ^{32}P-labeled ts 68-transformed cells incubated at 36°C with (*C* and *D*) and without (*A* and *B*) 5% calf serum.

serum at both temperatures and serum addition had little effect (data not shown). The phosphorylated S6 spots from these urea gels comigrated with the M_r 30,000 protein seen in whole cell extracts when cut out and run on $NaDodSO_4$ gels. Uptake of ^{32}P was the same in ts 68-infected cells at either temperature,

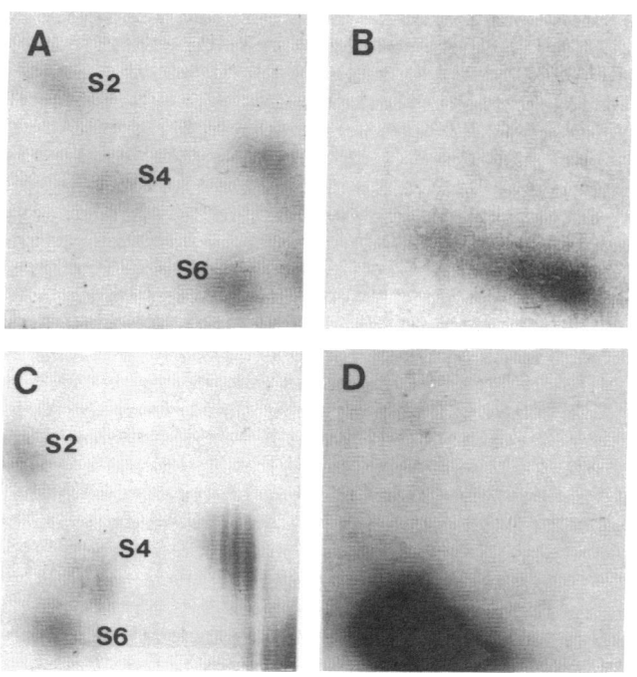

FIG. 4. Coomassie blue staining patterns (*A* and *C*) and autoradiograms (*B* and *D*) of two-dimensional urea gels of ^{32}P-labeled ts 68-infected cells incubated at 42°C with (*C* and *D*) and without (*A* and *B*) 5% calf serum.

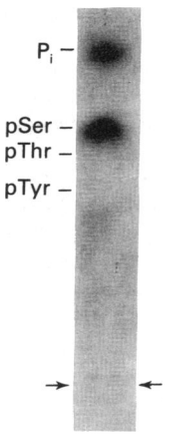

FIG. 5. Autoradiogram from high-voltage electrophoresis of acid hydrolysates of ^{32}P-labeled S6 ribosomal protein from ts 68-transformed cells incubated at 36°C. Positions of ninhydrin-stained phospho-amino acid standards (pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine) are indicated. Arrows, origin.

and ATP levels were unaffected by the temperature shift. Phospho-amino acid analyses of acid hydrolysates of the S6 bands showed that phosphoserine was the only phospho-amino acid detected. An autoradiogram of S6 hydrolysate from ^{32}P-labeled ts 68-transformed cells is shown in Fig. 5.

The results of attempts to quantitate the relative extent of phosphorylation of protein S6 from different cultures are shown in Table 1. They support the general trends suggested by the degree of anodal shift seen in stained gels, with an ≈6-fold increase in ^{32}P in serum-stimulated normal cells and in serum-stimulated ts 68-infected cells grown at the nonpermissive temperature. Serum addition had little effect on ^{32}P incorporation in ts 68-transformed cells incubated at 36°C or in SR-A-transformed cells. In an ASV-transformed rat cell line (RR 1022), the level of phosphorylation of S6 was also unaffected by the presence or absence of serum. Addition of epidermal growth factor (500 ng/ml) to serum-starved normal cells caused an appreciable increase in phosphate incorporation, as well as an anodal shift in the Coomassie blue staining spot (not shown). This effect is somewhat unexpected, as chicken embryo fibroblasts bind only small amounts of epidermal growth factor (S. Cohen, personal communication) and is perhaps due to the relatively high concentration used in these experiments. At 500–1000 ng/ml, epidermal growth factor stimulates thymidine incorporation in our chicken embryo fibroblast cultures (not shown).

DISCUSSION

This report confirms the observation that serum stimulates the phosphorylation of ribosomal protein S6 in normal chicken embryo fibroblasts (6). The change in mobility of the S6 protein on two-dimensional gels indicates that the phosphorylation occurs quantitatively and is not a consequence of alterations in the specific activity of ^{32}P-labeled ATP pools, which would lead to changes in the specific activity of S6-bound phosphate and to misinterpretation of data based strictly on ^{32}P assays.

Since many transformed cells show reduced requirements for serum or growth factors (10) and, indeed, many seem able to synthesize their own transformation-specific growth factors (18), it was of interest to determine the effects of removal of growth factors (i.e., serum starvation) on S6 phosphorylation in ASV-transformed cells. As shown, transformed cells acquired the capacity to phosphorylate S6 in the absence of serum (under conditions such that the unphosphorylated form of S6 was found

Table 1. Incorporation of ^{32}P into ribosomal protein S6

System	^{32}P, cpm per band	Ratio of stimulated to serum deprived
Normal cells without serum at 36°C	865	
Normal cells with serum at 36°C	5432	6.28
Normal cells with EGF (500 ng/ml) at 36°C	3328	3.85
ts 68-infected cells without serum at 36°C	5591	
ts 68-infected cells with serum at 36°C	6087	1.09
ts 68-infected cells without serum at 42°C	675	
ts 68-infected cells with serum at 42°C	4364	6.47
SR-A-infected cells without serum at 36°C	5291	
SR-A-infected cells with serum at 36°C	5587	1.06
SR-A-infected cells without serum at 42°C	4953	
SR-A-infected cells with serum at 42°C	5508	1.11
RR 1022 cells without serum at 36°C	3522	
RR 1022 cells with serum at 36°C	3793	1.08

^{32}P-Labeled S6 bands from first-dimension gels were assayed as described in *Materials and Methods*. Fifty micrograms of protein was applied to each lane.

in serum-starved normal cells). If indeed S6 phosphorylation functions in the translation of certain mRNAs involved in initiation of cell division (15), the ability of ASV-transformed cells to circumvent a normal cellular control mechanism by phosphorylating S6 independently of serum addition could be a factor contributing to their altered growth characteristics.

In addition, the fact that the ability of ts 68-infected cells to phosphorylate S6 in the absence of serum is temperature sensitive suggests that a functional *src* gene product is somehow involved in maintaining S6 in its phosphorylated form. This means that the transformed state, rather than virus multiplication, must be directly correlated to the phosphorylation of S6. However, the fact that the phosphorylation of S6 takes place at serine residues suggests that S6 phosphorylation is not due to direct action of the *src* gene product which phosphorylates tyrosine residues exclusively (14, 19, 20). Thus, it appears that the elevated phosphorylation of S6 in ASV-infected cells is a result of a transformation-specific alteration of metabolism that is induced and maintained by the *src* gene product.

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