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# Biochemical Characterization of a 34-Kilodalton Normal Cellular Substrate of pp60$^{v\text{-}src}$ and an Associated 6-Kilodalton Protein

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Transformation of fibroblasts by several retroviruses that produce transforming gene products associated with protein kinase activity results in the phosphorylation of a normal cellular protein with an $M_r$ of 34,000 (the 34K protein). Evidence is presented here that, as extracted from chicken embryo fibroblasts, this protein exists in two forms that differ both in their elution from hydroxylapatite and in their native molecular weight. The form that eluted from hydroxylapatite at 210 to 295 mM potassium phosphate displayed a native molecular weight of 30,000 to 40,000, whereas the form that eluted at 320 to 440 mM displayed a native molecular weight of 60,000 to 70,000. The latter form copurified with a low-molecular-weight protein with an approximate $M_r$ of 6,000 (6K). Both forms of 34K were completely separable from malate dehydrogenase activity. Phosphorylated 34K, isolated from Rous sarcoma virus-transformed cells, was also present in two forms; hence, in the cell neither form serves as a preferential substrate for pp60$^{v\text{-}src}$. We found that the expression of 34K differed greatly in various avian tissues. In particular, it was present in the highest concentration in cultured fibroblasts and in very low concentration in brain tissue. Its expression in this tissue seems to be controlled at the level of transcription, since 34K mRNA in brain tissue was barely detectable. The expression of 6K was similar to that of 34K.

Evidence has accumulated in several laboratories that avian sarcoma viruses encode transforming proteins that appear to function as protein kinases (for reviews, see references 18 and 19). Although it is not certain that this is the sole function of these proteins, it is one of the few clues available concerning the mechanism of transformation by these viruses, and therefore much effort has been expended to identify substrates for these virus-encoded protein kinases (8, 10–12, 16, 27, 32, 33, 35; for a review, see reference 23). The physiological significance of the transformation-specific phosphorylation of those cellular proteins alleged to be substrates remains to be determined, despite the fact that the normal function of these proteins is known in several cases.

To date, only one cellular protein found to be newly phosphorylated in avian sarcoma virus-transformed cells has also been shown to be directly phosphorylated in vitro with the same specificity by the Rous sarcoma virus transforming protein, pp60$^{v\text{-}src}$ (16). The function of this protein, variously assigned a molecular mass of 34 to 39 kilodaltons (kD) and here designated 34K, seems uncertain. It has been reported to be closely associated with malate dehydrogenase (MDH) (34; H. Rübsamen, P. Centner, E. Eigenbrodt, and R. R. Friis, Prog. Clin. Biol. Res., in press), although others have disputed this report (12, 13, 20). The biochemical evidence presented for either claim appears incomplete. There is also a report in the literature that this protein may function as an RNA-binding protein, since it is associated with ribonucleoprotein particles (2).

Most studies published on 34K have used parts of the purification protocol developed in this laboratory and described in a preliminary form in 1980 (16). Since that time, we have accumulated additional information about this protein that bears on a number of the outstanding issues, and we report these data in this communication.

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## MATERIALS AND METHODS

**Cells and viruses.** Chicken embryo fibroblasts were prepared from 11-day-old embryos (SPAFAS, Inc., Roanoke, Ill.). The Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A, was obtained originally from H. Hanafusa. Transformed chicken cells were used for experiments several passages after they had been infected. A stock of avian myeloblastosis virus was obtained originally from P. K. Vogt. Myeloblasts were obtained from leukemic birds by cardiac puncture (17). Cultures were radiolabeled with 50 to 100 μCi of [$^{35}$S]methionine (700 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml for 7 to 8 h in medium containing 1/10 the normal amount of methionine or with 0.5 to 1 mCi of $^{32}P_i$ (carrier-free; ICN Pharmaceuticals Inc., Irvine, Calif.) per ml for 2 h in phosphate-free medium. Cells were washed, scraped from the dishes, centrifuged, and stored at −70°C.

**Immunoprecipitation and polyacrylamide gel electrophoresis.** Proteins were resolved by electrophoresis through a discontinuous slab gel system (10% acrylamide, 0.26% bisacrylamide) with the buffer systems described by Laemmli (25). Acrylamide (2× crystallized) was from Serva (Serva Fine Biochemicals, Inc., Garden City Park, N.Y.), and bisacrylamide (Eastman Kodak Co., Rochester, N.Y.) was recrystallized from acetone. Before electrophoresis, samples were heated at 95°C for 1 min in electrophoresis sample buffer (70 mM Tris-hydrochloride [pH 6.8], 11% glycerol, 3% sodium dodecyl sulfate [SDS], 0.01% bromophenol blue, 5% 2-mercaptoethanol). Gels were stained with 0.2% Coomassie blue in 50% trichloroacetic acid and destained in 10% acetic acid–5% methanol. A mixture of molecular weight standards for SDS-polyacrylamide gel electrophoresis, purchased from Sigma Chemical Co., St. Louis, Mo., contained β-galactosidase (*Escherichia coli*; $M_r$ = 116,000), phosphorylase *b* (rabbit muscle; $M_r$ = 97,400), bovine albumin ($M_r$ = 66,000), ovalbumin ($M_r$ = 45,000), and carbonic anhydrase (bovine erythrocyte; $M_r$ = 29,000). $^{32}$P-labeled proteins were

visualized by autoradiography with the aid of Du Pont Lightning-Plus intensifying screens. Fluorography with sodium salicylate (6) was used to enhance the detection of $^{35}S$-labeled proteins.

Immunoprecipitations were carried out with the use of protein A-bearing *Staphylococcus aureus* (24). For immunoprecipitation, cells were lysed in buffer containing 100 mM NaCl, 10 mM Tris (pH 7.2), 1 mM EDTA, 1% Nonidet P-40 (NP-40), and 0.5% sodium deoxycholate and clarified at 100,000 × *g* for 30 min. Samples from the various fractionation procedures were diluted with 0.5 ml of the buffer described above before the addition of antiserum. The bacteria-immune complexes were washed several times as described previously (15), and the immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis. The preparation of anti-34K serum and its initial characterization have been described (15). Radiographic detection of antigens was also carried out by the "Western blotting" technique as described by Burnette (5), except that the final washes contained 0.05% Triton X-100.

**Isolation of RNA and in vitro translation.** RNA was isolated from normal chicken embryo fibroblasts and from chicken brain by SDS-phenol-chloroform-isoamyl alcohol extraction, and the polyadenylate-containing RNA was selected by oligodeoxythymidylate cellulose chromatography as described previously (4). In vitro translations were carried out in a messenger-dependent reticulocyte system (31) with the ionic conditions described by Erikson et al. (14). The products of in vitro translation were resolved by SDS-polyacrylamide gel electrophoresis both directly and after immunoprecipitation.

**Other analyses.** For two-dimensional peptide fingerprinting, polypeptides were eluted from preparative gels, precipitated, and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (7). The peptides were resolved by ascending chromatography in *n*-propanol-*sec*-butanol-isoamyl alcohol-pyridine-water (1:1:1:3:3) in the first dimension and by electrophoresis at pH 3.5 (pyridine-acetic acid-water [1:10:189]) in the second dimension.

Protein concentration was determined by the method of Lowry et al. (26). Radioactivity was quantified by liquid scintillation spectrometry. A liquid sample was spotted onto Whatman GFA glass fiber paper, dried, and counted with PPO (2,5-diphenyloxazole)-dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene]-containing scintillation fluid. For estimation of radioactivity in a protein band in a fluorographed gel, the pertinent region was excised and counted directly with scintillation fluid. This method is less efficient than with soluble samples, but it gives a linear relationship compared with the intensity of the corresponding film.

MDH assays were carried out in the presence of 100 mM sodium phosphate (pH 7.5), 0.5 mM oxaloacetate, and 0.2 mM NADH (3). The reverse reaction was carried out in the presence of 0.4 M hydrazine, 0.5 M glycine (pH 9.5), 0.5 mM malate, and 0.3 mM $NAD^+$ (22).

**Fractionation of the 34-kD protein.** Normal chicken embryo fibroblasts (600 to 800 mg [wet weight]) biosynthetically radiolabeled with [$^{35}S$]methionine were lysed in 17 ml of 10 mM Tris-hydrochloride (pH 7.2)–1 mM EDTA–1 mM 2-mercaptoethanol–0.05% NP-40 (buffer A minus glycerol) with 25 strokes in a Dounce homogenizer. After clarification at 100,000 × *g* for 30 min, glycerol was added to a final concentration of 10% (buffer A), and the supernatant solution was passed through a column (13 by 42 mm) of DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated in buffer A at a flow rate of 1 ml/4 min. The column was washed with buffer A until the breakthrough fractions had been collected, and this material was loaded onto a column of hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) (13 by 70 mm) equilibrated in buffer A. The column was washed with 20 ml of buffer A and then eluted with a total volume of 150 ml of a linear gradient of 0 to 500 mM potassium phosphate in 1 mM EDTA–1 mM 2-mercaptoethanol–0.05% NP-40–10% glycerol at a flow rate of 1 ml/2.2 min. Fractions of 2 ml were collected. The ionic strength of the solutions was estimated from the refractive index.

The fractions of interest were pooled, dialyzed against buffer A, and fractionated a second time on hydroxylapatite as described above. The fractions of interest were pooled, dialyzed against buffer A, concentrated by adsorption to and elution from a 1-ml column of hydroxylapatite, dialyzed against 50% glycerol in buffer B (buffer A minus NP-40) and then against buffer B. Gel filtration was carried out on a Sephacryl S-200 (Pharmacia) column (2.5 by 28 cm) equilibrated in buffer B at a flow rate of 1 ml/min. Fractions of 1 ml were collected.

Sedimentation analysis was performed through glycerol gradients (30 to 10%) in 150 mM NaCl–50 mM Tris (pH 7.5)–0.1 mM EDTA–1 mM dithiothreitol–0.05% NP-40. Proteins used as molecular weight standards for gel filtration or sedimentation were as follows: urease (jack bean; $M_r$ = 483,000, for the determination of $V_0$), alcohol dehydrogenase (yeast; $M_r$ = 145,000), peroxidase (horseradish; $M_r$ = 42,000), and cytochrome *c* (horse heart; $M_r$ = 13,000), all from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

## RESULTS

**Separation of two forms of the 34-kD protein by ion-exchange chromatography.** As detailed above, a cellular lysate was clarified, passed through DEAE-Sephacel, and then fractionated on hydroxylapatite. Figure 1 displays the elution pattern of [$^{35}S$]methionine-labeled proteins from the hydroxylapatite column. Numerous proteins with an $M_r$ of 30,000 to 40,000 were eluted from the column in fractions 25 to 70. The fractions in the region from 320 to 440 mM potassium phosphate (fractions 52 to 68) were shown originally to contain the 34-kD protein of interest by analysis with the two-dimensional procedure of O'Farrell et al. (16, 30). A protein preparation such as this was recycled through a second hydroxylapatite column (where it again eluted between 320 and 440 mM potassium phosphate), subjected to gel filtration through Sephacryl S-200, and then used to prepare antibody as described previously (15, 16). At that time, the purity of the preparation used as antigen was examined by Coomassie blue staining of SDS-polyacrylamide gels and of two-dimensional gels (16). The resulting antiserum was analyzed by examination of immunoprecipitates both by SDS-polyacrylamide gel electrophoresis and by the two-dimensional technique (15) and also by Western blotting (5) of cellular extracts that had been fractionated on a 5 to 15% SDS-polyacrylamide gel.

Since there were many proteins in the 30- to 40-kD range in the eluate from the hydroxylapatite column (Fig. 1), anti-34K serum was used to determine whether any of these proteins were antigenically related to the 34-kD protein that eluted in fractions 52 to 68 and that had been used to elicit antibody. In addition to the 34-kD protein that eluted at 320 to 440 mM potassium phosphate, a protein with an $M_r$ of 34,000 that eluted earlier from the column at 210 to 295 mM potassium phosphate was specifically immunoprecipitated

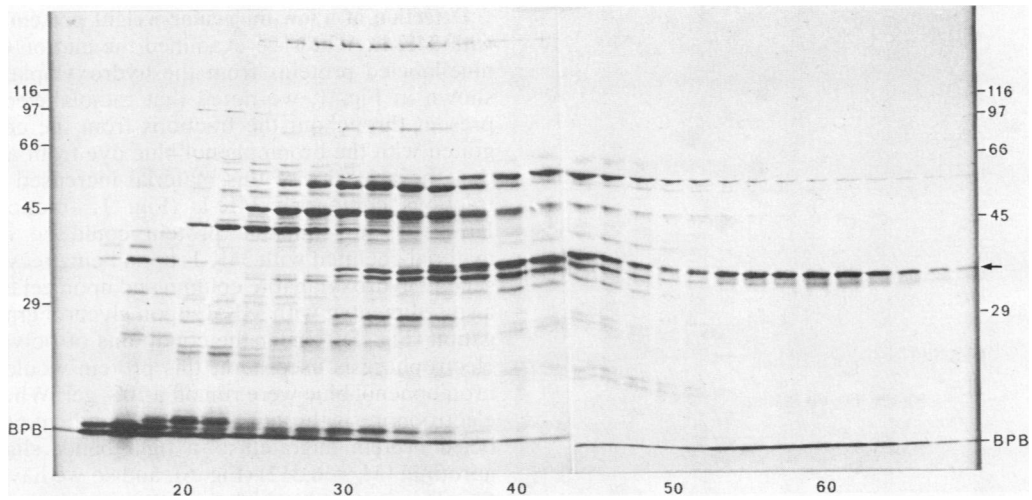


FIG. 1. Fractionation of proteins on hydroxylapatite. A lysate from [$^{35}$S]methionine-labeled cells was clarified, passed through DEAE-Sephacel, and then loaded onto hydroxylapatite. The column was eluted with a 0 to 500 mM gradient of potassium phosphate as described in the text. The gradient elution started with fraction 1 and continued until fraction 80; 20-μl samples of the even-numbered fractions were subjected to polyacrylamide gel electrophoresis. The ordinates indicate the position of the molecular weight standards ($\times 10^3$), which were adjusted to the same buffer conditions as the pertinent samples and loaded into the end wells of each gel. The position of the bromophenol blue dye (BPB) is also indicated. The arrow indicates the 34-kD protein.

by anti-34K serum (Fig. 2). Upon being recycled through a second hydroxylapatite column, this protein again eluted at 210 to 295 mM potassium phosphate. To determine whether these two 34-kD proteins were structurally identical, tryptic peptide maps were compared. These 34-kD proteins yielded identical peptide fingerprints (Fig. 3). Analysis of a mixture confirmed that the fingerprints were identical (data not shown). Therefore, it is likely that these are two forms of the same protein that, possibly because of association with other proteins or conformational differences, are separated by hydroxylapatite chromatography. The two forms of the 34-kD protein are designated E (early) and L (late), in order of their elution from hydroxylapatite. The distribution of 34K between these two forms was usually 25 to 30% E and 70 to 75% L.

Since 34K is phosphorylated in vivo in a transformation-dependent manner, we analyzed the fractionation of the phosphorylated form of this protein by the same procedures. Phosphorylated 34K also eluted from the hydroxylapatite column in two peaks, each of which eluted in its original position upon recycling (data not shown). Tryptic phosphopeptide maps of these preparations were identical (Fig. 3).

**Separation of the 34-kD proteins from MDH.** It has been reported that 34K is MDH (34; Rübsamen et al., in press). We have not been able to demonstrate significant MDH activity in our preparation of 34K-L, prepared as described originally (16). In the experiments reported by Rübsamen et al., however, the hydroxylapatite column used was much smaller than the one described in Fig. 1, and it was eluted in a different manner. Since we now have shown that some 34K exists in a form that elutes early from hydroxylapatite, it is possible that this form of the protein has MDH activity or that this form coeluted or overlapped the elution of MDH from the hydroxylapatite column in the work cited above. Therefore, the distribution of MDH activity in the fractions from the hydroxylapatite column was monitored. We found that the majority of the MDH activity eluted between 120 and 210 mM potassium phosphate (Fig. 2) and that less than 5% was present in the fractions that contained 34K-E or 34K-L. Similar results were obtained when MDH activity was assayed by the reverse reaction. Moreover, no 34-kD protein was detectable by immunoprecipitation in the fractions containing MDH activity (Fig. 4). The distribution and recovery of 34K and of MDH activity are given in Table 1.

**Estimation of the native molecular weights of the two forms of 34K.** We reported previously that upon gel filtration 34K, in that case the L form, eluted near the region of hemoglobin, and therefore we suggested that it may exist as a dimer

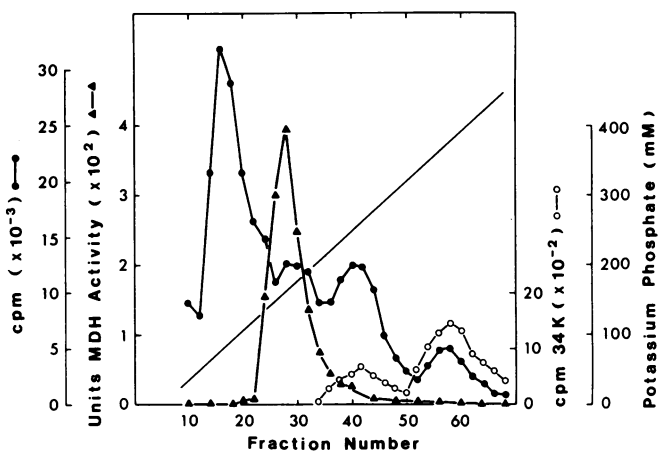


FIG. 2. Distribution of immunoprecipitable proteins with an $M_r$ of 34,000 and of MDH activity in the fractions from hydroxylapatite chromatography. From the indicated fractions of the hydroxylapatite column shown in Fig. 1, 20-μl samples were spotted onto glass fiber paper for determination of total radioactivity. Samples (20 μl) were diluted with 0.5 ml of buffer and immunoprecipitated with 3 μl of anti-34K serum as detailed in the text. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis. The gels were fluorographed, the pertinent bands were excised, and the radioactivity was determined. Symbols: ●, total radioactivity; ○, immunoprecipitated 34-kD protein. In addition, 45-μl samples from the even-numbered fractions of the column were assayed for MDH activity. When the peak of activity had been located, 5-μl samples of the appropriate fractions were reassayed. ▲, MDH activity (IU/45 μl).

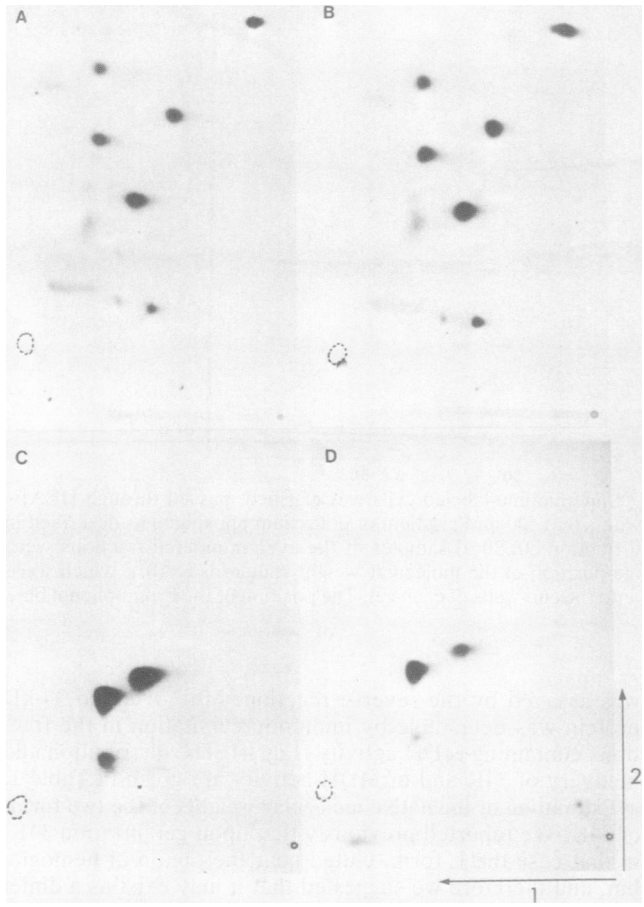


FIG. 3. Tryptic peptide fingerprints of the two proteins with an $M_r$ of 34,000. The fractions from the hydroxylapatite column that contained the two proteins with an $M_r$ of 34,000 detected by immunoprecipitation (fractions 35 to 46 and 52 to 68) were pooled separately, dialyzed against buffer A, and recycled through second hydroxylapatite columns. The appropriate fractions were pooled and immunoprecipitated, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis. The pertinent regions of the gel were excised, the proteins were eluted, and their tryptic peptides were fractionated as described in the text. Tryptic phosphopeptide maps were prepared in a similar manner from Rous sarcoma virus-transformed chicken embryo fibroblasts that had been radiolabeled in culture with $^{32}P_i$. (A) [$^{35}$S]methionine-labeled 34K-L; (B) [$^{35}$S]methionine-labeled 34K-E; (C) $^{32}$P-labeled 34K-L; (D) $^{32}$P-labeled 34K-E. The dashed circle indicates the position of ε-2,4-dinitrophenyl-lysine.

or have an unusual structure (16). Accordingly, 34K-L and 34K-E were subjected to gel filtration through Sephacryl S-200, as detailed above. 34K-L eluted from the column in a position consistent with a molecular weight of 65,000 to 70,000. Under similar conditions, 34K-E eluted from the column later than 34K-L, in a manner consistent with a molecular weight of approximately 40,000. However, under the conditions selected, there was significant trailing, and recovery was poor. Therefore, these protein preparations were also subjected to glycerol gradient sedimentation analysis. When examined by this method, 34K-L again behaved as though it had a larger size than 34K-E (Fig. 5). The molecular weights estimated from this procedure were 60,000 to 70,000 and 30,000 for the L and E forms, respectively.

**Detection of a low-molecular-weight protein that copurifies with 34K-L.** When we examined the elution of [$^{35}$S]methionine-labeled proteins from the hydroxylapatite column, as shown in Fig. 1, we noted that radiolabeled material was present throughout the fractions from the column that migrated with the bromophenol blue dye front and, moreover, that the intensity of this material increased slightly in the fractions containing 34K-L (Fig. 1; fractions 52 to 68). However, no stainable protein could be detected. This material coeluted with 34K-L upon being recycled through a second hydroxylapatite column and upon gel filtration, and it also sedimented with 34K-L upon glycerol gradient centrifugation (Fig. 5). Under the conditions of polyacrylamide gel electrophoresis used here, this protein would be lost if the bromophenol blue were run off a 10% gel. When subjected to electrophoresis through a 5 to 15% gradient gel, this radiolabeled protein migrated as a tight band, slightly ahead of aprotinin ($M_r$ = 6,512) (Fig. 6), and so we have designated it 6K. This material, which had not been detected previously, was present in the antigen used to prepare the anti-34K serum, and it is possible that antibody had been generated against this protein also. Therefore, we reexamined the anti-34K serum for anti-6K activity.

Although analysis of our anti-34K serum by Western blotting did not disclose any radiolabel in the 6-kD region, treatment of extracts of [$^{35}$S]methionine-labeled cells with anti-34K serum specifically did immunoprecipitate radiolabeled material that migrated with the dye front in a 10% gel

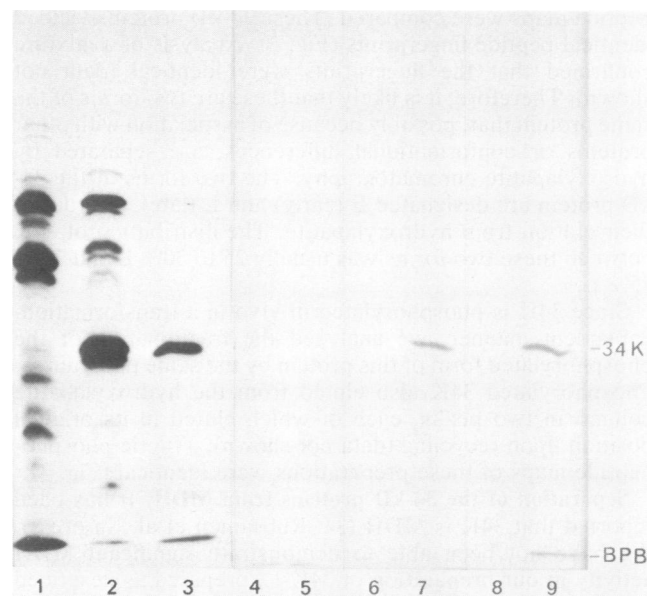


FIG. 4. Immunoprecipitation of various fractions from hydroxylapatite chromatography. The fractions from the hydroxylapatite column that contained the peak of MDH activity (fraction 28), 34K-E (fraction 42), and 34K-L (fraction 58) were analyzed by SDS-polyacrylamide gel electrophoresis both directly and after immunoprecipitation with anti-34K serum. Ten-microliter samples were analyzed directly; 20-μl samples of fractions 42 and 58 and 100-μl samples of fraction 28 were immunoprecipitated. Lanes 1, 2, and 3, samples from fractions 28, 42, and 58 analyzed directly; lanes 4 and 5, immunoprecipitated samples from fraction 28; lanes 6 and 7, immunoprecipitated samples from fraction 42; lanes 8 and 9, immunoprecipitated samples from fraction 58. Lanes 4, 6, and 8, preimmune serum; lanes 5, 7, and 9, anti-34K serum. Lanes 4 and 5 were exposed three times longer than the other lanes. BPB, Bromophenol blue.

TABLE 1. Fractionation of the 34-kD protein and of MDH activity upon ion-exchange chromatography[a]

| Fractionation step | Protein | | 34K (cpm) | MDH (IU) | |
|---|---|---|---|---|---|
| | mg | cpm[b] | | a | b |
| 100,000 × *g* pellet | | | $13 \times 10^4$ | | |
| 100,000 × *g* supernatant | 52 | $5.8 \times 10^8$ | $74 \times 10^4$ | 33.8 | 0.38 |
| DEAE breakthrough | 16.5 | $1.2 \times 10^8$ | $74 \times 10^4$ | 18.9 | 0.20 |
| Hydroxylapatite | | | | | |
| (120 to 210 mM; fractions 23 to 34) | 3.6 | $1.9 \times 10^7$ | $<1 \times 10^3$ | 19.2 | 0.18 |
| (210 to 295 mM; fractions 35 to 46) | 3.4 | $1.4 \times 10^7$ | $29 \times 10^4$ | 0.69 | 0.01 |
| (320 to 440 mM; fractions 52 to 68) | | $0.8 \times 10^7$ | $82 \times 10^4$ | 0.65 | (Undetectable) |

[a] The amount of 34K in the different samples was determined by immunoprecipitation of various amounts with a constant amount of antiserum. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis, the 34-kD regions of the gel were excised, and the radioactivity was quantitated. The total amount of radioactivity in the 34-kD protein in a particular sample was calculated from the immunoprecipitations from which the amount of 34K recovered was linear with the amount of lysate used. For estimation of the amount of 34K in the pellet from the 100,000 × *g* clarification, the pellet was resuspended in 0.1% SDS and reclarified, and samples of this supernatant were immunoprecipitated. MDH activity was assayed in both the (a) oxaloacetate → malate and (b) malate → oxaloacetate directions.

[b] Determined from a sample spotted onto glass fiber paper.

(Fig. 7). To address the issue of whether this protein was immunoprecipitated because of specific antibody against it, because of its possible association with 34K-L, or because of structural relatedness to 34K, the following experiments were carried out. The 6-kD protein was eluted from a preparative gel and subjected to reimmunoprecipitation with anti-34K serum. This protein was immunoprecipitated by anti-34K serum even in the absence of 34K. The reason for the previous lack of detection of this antibody by the Western technique was investigated by monitoring a preparation of [$^{35}$S]methionine-labeled 34K-L at several stages of the Western procedure and we found that, although the 6-kD protein was electrophoresed out of the gel and was adsorbed fairly efficiently to the nitrocellulose, it was lost almost completely during the incubation and washing steps (Fig. 7). Tryptic fingerprints of the two proteins were compared and did not indicate any common methionine-containing peptides in 34K and 6K (Fig. 8). Nor did analysis of chymotryp-

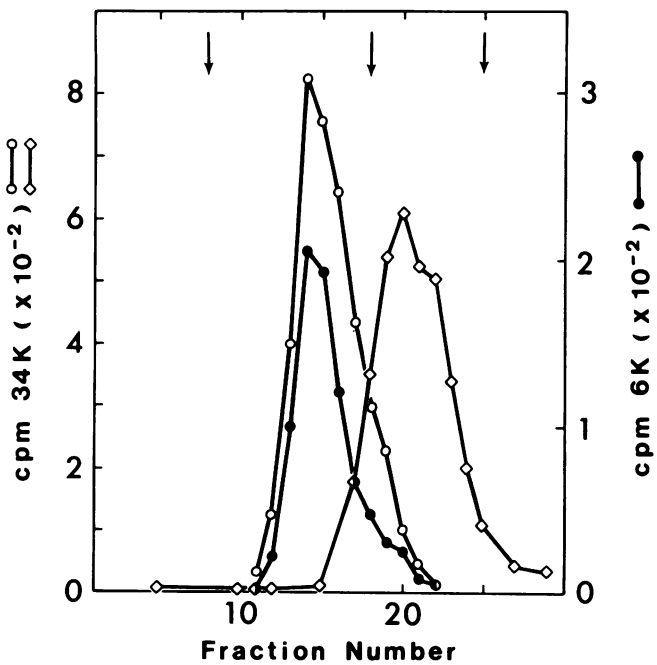


FIG. 5. Sedimentation analysis of the two forms of 34K. A preparation of 34K-L that had been recycled through a second hydroxylapatite column was concentrated, and a sample was subjected to glycerol gradient centrifugation as described in the text. Fractions of approximately 160 μl were collected, and 20-μl samples of the indicated fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The pertinent bands were excised from the gel, and the radioactivity was determined. A preparation of 34K-E was concentrated, and a sample was subjected to glycerol gradient centrifugation. Fractions of 160 μl were collected, and 20-μl samples were immunoprecipitated with anti-34K serum and analyzed as described above. Sedimentation was from right to left. The positions of the protein standards are indicated by arrows: alcohol dehydrogenase, fraction 8; horseradish peroxidase, fraction 18; cytochrome *c*, fraction 25. Symbols: ○, 34K-L; ◇, 34K-E; ●, 6K.

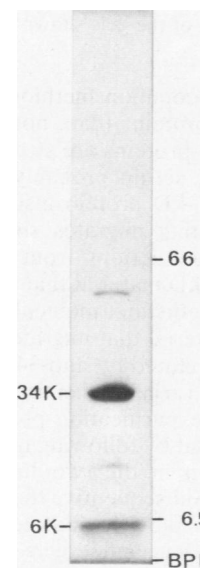


FIG. 6. Analysis of a 34K-L preparation by electrophoresis through a 5 to 15% gradient gel. A sample of a [$^{35}$S]methionine-labeled preparation of 34K-L, such as that shown in Fig. 1, lane 58, was subjected to electrophoresis through a 5 to 15% SDS-polyacrylamide gel. The right ordinate indicates the position of bovine albumin and aprotinin (×10$^3$). BPB, Bromophenol blue.

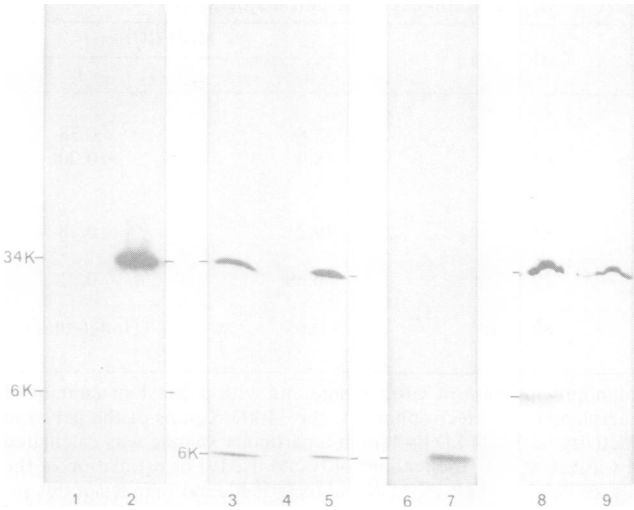


FIG. 7. Demonstration of anti-6K activity in anti-34K serum. Normal chicken embryo fibroblasts were lysed and clarified, and 50 μg of protein per well was subjected to SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose (BA 85, 0.45 μm; Schleicher & Schuell Co., Keene, N.H.) and reacted with antiserum and $^{125}$I-protein A as described by Burnette (5). Lane 1, preimmune serum; lane 2, anti-34K serum. The positions of the 34-kD and 6-kD proteins were determined by parallel transfer of a [$^{35}$S]methionine-labeled preparation, such as that shown in lanes 3 and 8. A lysate of [$^{35}$S]methionine-labeled cells was immunoprecipitated, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis. Lane 3, [$^{35}$S]methionine-labeled 34K and 6K to serve as markers; lane 4, preimmune serum; lane 5, anti-34K serum. The [$^{35}$S]methionine-labeled 6-kD protein was eluted from a preparative gel, such as that shown in lane 5, and reimmunoprecipitated. Lane 6, preimmune serum; lane 7, anti-34K serum. A [$^{35}$S]methionine-labeled preparation of 34K and 6K, such as that shown in lane 3, was electrotransferred to nitrocellulose. One nitrocellulose strip was not processed further (lane 8), and the other (lane 9) was subjected to the incubations and washes of the Western procedure, except that no radioiodinated protein A was added. The position of 6K relative to 34K is different in the various lanes because of the expansion of the gels shown in lanes 3 through 7.

tic digests reveal any common methionine-containing peptides from these two proteins (data not shown). Thus, it is unlikely that these two proteins are structurally similar, and therefore the anti-34K serum probably contains a distinct antibody against the 6-kD protein also. Although there is radiolabeled material that migrates similarly to this 6-kD protein in most of the fractions from the hydroxylapatite column (Fig. 1), the 6-kD material that copurified with 34K-L seems to represent a distinct molecular species, since the other radiolabeled material that migrated with the dye front was not immunoprecipitated by anti-34K serum (Fig. 4).

**Expression of 34K in various avian tissues.** As stated above, the initial steps of the purification protocol for the 34-kD protein were worked out by following the $^{32}$P-labeled protein based on its migration in the two-dimensional system of O'Farrell et al. (30). Subsequently, the fractionation of the protein could be followed by Coomassie blue staining of one-dimensional SDS-polyacrylamide gels. Initial efforts to obtain this protein by applying the same protocol to chicken embryos or to commercially available tissues were not successful. When anti-34K serum became available, we were able to quantitate the content of 34K in a variety of tissues, both to obtain a source of material more plentiful than cultured chicken embryo fibroblasts and also to obtain information as to the possible function of this protein. To this end, immune competition experiments were carried out with extracts of several tissues and extracts of [$^{35}$S]methionine-labeled chicken embryo fibroblasts. We found that the expression of this protein varied by more than 100-fold, with fibroblasts showing the highest amount. 34K was not detectable in erythrocytes and was present in very low amounts in brain tissue. In addition, we found that myeloblasts had a high content of this protein, approximately one-tenth that of fibroblasts. These data are presented in Fig. 9. The content of 34K in brain tissue was also quantified by the Western blotting technique, and the results substantiated those obtained by the immune competition experiments (data not shown). As judged by the specific radioactivity in the region of the 34-kD protein, brain tissue contained less than 5% of the level of the 34-kD protein present in fibroblasts. Greenberg and Edelman (20) have also reported that various tissues contain a lower concentration of 34K than fibroblasts.

The content of 6K in the various tissues was also determined by immune competition, and we found that its expression was similar to that of 34K (data not shown).

**Expression of 34K mRNA in chicken brain and chicken embryo fibroblasts.** To ascertain at what level the expression of 34K was controlled in brain tissue, we examined the products of in vitro translation of mRNA prepared from this tissue. Figure 10 illustrates the results of immunoprecipitation of polypeptides synthesized in cell-free extracts programmed by brain mRNA and chicken embryo fibroblast mRNA. It is clear that the level of 34K mRNA in brain was almost undetectable by this procedure. As estimated from the amount of radioactivity in the 34-kD region of the immunoprecipitates, the level of 34K mRNA in brain is less than 5% of that in fibroblasts. In a similar manner, we estimate that chicken embryos contain approximately 20% of the level of 34K mRNA present in fibroblasts.

## DISCUSSION

In this communication, we describe further details on the purification of the 34-kD protein substrate for pp60$^{v\text{-}src}$ that we had partially characterized in an earlier study (16). Our data show that there are at least two relatively stable forms of the protein released from cells. These forms can be separated by hydroxylapatite chromatography and have been designated E and L, in order of their elution. In addition, the two forms differ in their native

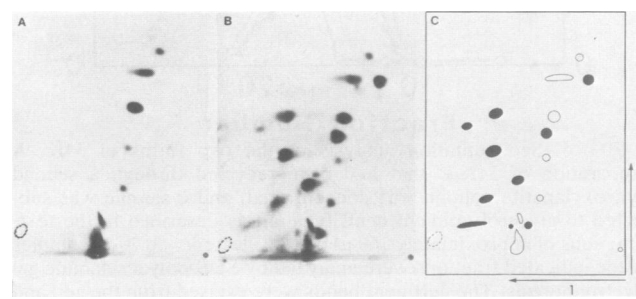


FIG. 8. Tryptic peptide fingerprints of 34K and 6K. [$^{35}$S]methionine-labeled 34K and 6K were eluted from a preparative gel, such as that in Fig. 7, lane 5, and the tryptic peptides were resolved as described in the text. (A) 6K; (B) mixture of 34K and 6K; (C) schematic diagram showing the peptides originating from 34K (●) and from 6K (○). The dashed circle indicates the position of ε-2,4-dinitrophenyl-lysine. A tryptic peptide fingerprint of 34K is shown in Fig. 3A.

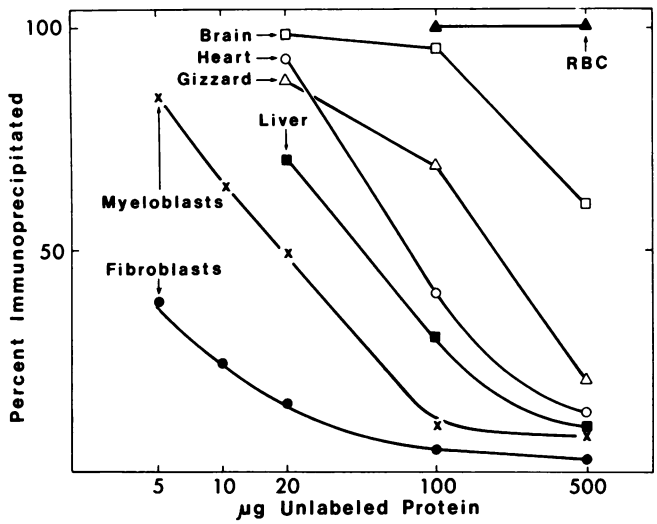


FIG. 9. Quantification of 34K in several tissues. The indicated organs were removed from a 6-week-old chicken, and myeloblasts were collected from a leukemic chicken. Lysates were prepared from these tissues and from cultured chicken embryo fibroblasts, and samples containing from 5 to 500 μg of soluble protein were incubated for 1 h with 1 μl of anti-34K serum. Then, samples of a lysate prepared from [$^{35}$S]methionine-labeled chicken embryo fibroblasts were added, and the immunoprecipitated proteins were analyzed by polyacrylamide gel electrophoresis. The 34K bands were excised, and the radioactivity was determined by liquid scintillation spectrometry. The 34K content of the various lysates is reflected by the ability of that lysate to block the immunoprecipitation of the radiolabeled protein. RBC, erythrocytes.

molecular weights. Upon gel filtration or glycerol gradient sedimentation, 34K-E exhibited a molecular weight of 30,000 to 40,000. Therefore, under these conditions it is apparently a monomer and is not associated with any of the other proteins that elute similarly from hydroxylapatite. In contrast, 34K-L displayed a native molecular weight of 60,000 to 70,000, and thus, as we suggested previously (16), it may exist as a dimer in its native form.

It is possible that the subcellular location of the two forms of the 34-kD protein differs or, alternatively, that the two forms may be interchangeable in the cell in a manner that relates to its function, and this state may be frozen by the conditions used for cell lysis. Numerous investigators have reported that in cell fractionation studies most of the 34-kD protein is found with crude membranes and a large percentage with the plasma membranes (1, 9, 13, 21). However, these studies also revealed that under certain conditions a significant portion of 34K was either in the soluble fraction or only peripherally associated with the plasma membranes. In addition, although immunofluorescence studies indicate 34K to be located at the cytoplasmic face (13, 21, 28), analysis of radiolabeled cells subjected to the immunofluorescence procedure showed that as much as 50% of 34K had been extracted (28). This behavior of 34K may be due to the partitioning of the two forms of the protein, but additional experiments are required to ascertain whether or not this is the case. The phosphorylated 34-kD protein also fractionated into the E and L forms, suggesting that neither form is preferentially available as a pp60$^{v\text{-}src}$ substrate.

A variety of evidence suggests that the late fractions from the hydroxylapatite column contain no proteins in the molecular weight range of 34,000 other than the pp60$^{v\text{-}src}$ substrate (16). This is not the case for the early fractions, where there are at least three or more distinct proteins with similar molecular weights as judged by SDS-polyacrylamide gel electrophoresis. This latter observation may clarify the origin of the MDH activity reported previously to be associated with 34K (34; Rübsamen et al., in press). If the hydroxylapatite column were eluted with steps of phosphate buffer or a steep gradient, it is possible that the enzymatic activity and 34K would appear to coelute. However, gradient elution, as performed in the experiments described here, clearly resolves MDH activity from even the early form of the 34-kD protein, suggesting that two distinct proteins are involved. In this regard, it is relevant that avian brain tissue has very low levels of 34K, whereas the level of MDH activity in this tissue is equivalent to that found in fibroblasts (H. G. Tomasiewicz, unpublished data). These various results together argue that 34K, the pp60$^{v\text{-}src}$ substrate, is not MDH.

In addition, the data presented here demonstrate the copurification of a protein with an $M_r$ of 6,000 with 34K-L. This 6-kD protein went undetected in our previous reports (15, 16) because it moves at the front of a 10% polyacrylamide gel, does not stain well with Coomassie blue, and apparently is readily eluted from gels during destaining because of its low molecular weight. Since the column fractions containing the 34-kD protein used for preparation of anti-34K serum also contained the 6-kD protein, serum raised against 34K also contained anti-6K activity. This activity could not be detected by Western blotting, because, although 6K was transferred to the nitrocellulose, it was eluted during the subsequent washing procedures. The possible presence of anti-6K activity in anti-34K sera prepared by a similar or related protocol should be considered.

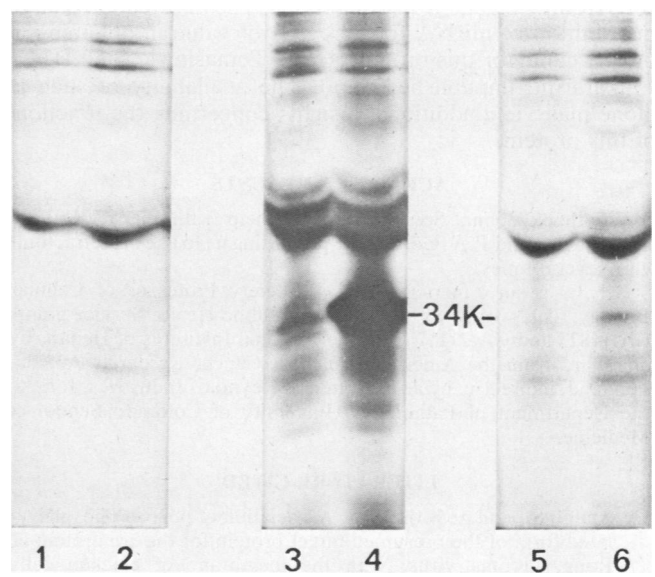


FIG. 10. Immunoprecipitation of the products of in vitro translation. The RNA used to program the lysate and the serum used for immunoprecipitation were as follows: lane 1, no RNA added, preimmune serum; lane 2, no RNA added, anti-34K serum; lane 3, chicken embryo fibroblast mRNA, preimmune serum; lane 4, chicken embryo fibroblast mRNA, anti-34K serum; lane 5, brain mRNA, preimmune serum; lane 6, brain mRNA, anti-34K serum. The 0.3 M KOH-resistant and 10% trichloroacetic acid-precipitable radioactivity above background was similar for both mRNAs, and the range of sizes of the translation products, as analyzed directly by SDS-polyacrylamide gel electrophoresis, was also similar (data not shown).

It is possible that the 34-kD and 6-kD proteins may be components of the same structure, although the evidence shown here does not firmly establish such a relationship. Since 34K-L exhibits a native molecular weight of 60,000 to 70,000, it may exist as a dimer, or as a dimer in association with one or a few 6-kD proteins. The stoichiometry of 34K and 6K in the 34K-L preparation has not yet been determined with precision because of the possible loss of some 6K from the gel and incomplete recovery of 6K by trichloroacetic acid precipitation. Because 6K also exhibits a native molecular weight of 60,000 to 70,000, if it were not associated with another protein(s), it would have to exist as a multimer. Whether the 6-kD protein plays a role in the function of 34K or in the modulation of that function remains to be determined. In this regard, it is of interest that the expression of 6K in the various avian tissues examined parallels that of 34K. The level of phosphorylation of 6K, which was barely detectable, was the same in normal or transformed cells. Additional definition of the possible interactions of 6K and 34K may provide leads to other cellular components that comprise a protein network upon which $pp60^{v\text{-}src}$ impinges.

Others (36) have reported that 34K is not detected in some transformed B-lymphocyte populations and have suggested that this protein plays a role in cell adhesion or migration on a substratum. Our results showing 34K present at significant levels in avian myeloblastosis virus-transformed myeloblasts are of interest in this regard because these cells would not normally exhibit such behavior in the absence of further differentiation. Clearly, additional studies on cells of bone marrow origin would be of value.

The control of expression of the 34-kD protein seems to be at the level of transcription since only low levels of mRNA for 34K are detectable in brain tissue. The information on the distribution of mRNA for 34K was of value in obtaining a cDNA clone for this protein (H. G. Tomasiewicz and D. L. Chikaraishi, unpublished data). The availability of such a clone may yield additional insights concerning the function of this protein.

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