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PHOSPHORYLATION OF THE M_r = 34,000 PROTEIN IN NORMAL AND ROUS SARCOMA VIRUS-TRANSFORMED RAT FIBROBLASTS

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Summary: Antiserum was raised against the M_r = 34,000 chick cell protein which may serve as a substrate for the Rous sarcoma virus transforming gene product. The antiserum specifically immunoprecipitated 2 proteins from [^{35}S]methionine labeled Rous sarcoma virus-transformed rat cell extracts (a M_r = 35,000 and a M_r = 38,000 protein). Partial protease treatment revealed these two proteins to be very closely related. The protein of apparent M_r = 38,000 was phosphorylated and the phosphate was present exclusively on tyrosine residues. The effect of epidermal growth factor on phosphorylation of the M_r = 35,000 protein was examined in several normal rat fibroblast cell lines. EGF treatment had no effect on phosphorylation of the M_r = 35,000 protein for any normal cell line and also failed to elevate overall levels of phosphotyrosine.

A 34,000 - 36,000 molecular weight chick cell protein becomes rapidly phosphorylated on tyrosine residues when cells transformed by temperature sensitive mutants of Rous sarcoma virus are shifted from the non-permissive to permissive temperature (1). This protein is also phosphorylated *in vitro* by purified Rous sarcoma virus transforming gene product ($pp60^{src}$) (2) and is a likely *in vivo* substrate for $pp60^{src}$ which possesses protein kinase activity. A similar protein is phosphorylated in the human epidermoid carcinoma A431 cell line in response to administration of EGF (3, 4). In this study the M_r = 34,000 protein from a variety of normal and Rous sarcoma virus-transformed rat cell lines was found to cross-react with antiserum prepared against the M_r = 34,000 chick cell protein. The effects of Rous sarcoma virus-transformation and EGF treatment on phosphorylation of this protein were examined.

Abbreviations: EGF: epidermal growth factor; DMEM: Dulbecco's modified Eagle's medium; SDS: sodium lauryl sulfate.

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Materials and Methods

Cells. RAT-1 cells, 3Y1 cells, and Rous sarcoma virus Schmidt-Ruppin strain subgroup A transformed 3Y1 cells were obtained from H. Hanafusa (Rockefeller University). Normal rat kidney fibroblast clone 49F cells and A431 epidermoid carcinoma cells were kindly provided by J. DeLarco (NIH). The Rous sarcoma virus transformed rat cell line RR 1022 was from A. Goldberg (Rockefeller University). All cells were grown in DMEM containing 10% calf serum.

Radioactive Labeling. Subconfluent cells were labeled with [^{35}S]methionine (50 μCi/ml) (New England Nuclear, Boston, MA) in DMEM lacking methionine containing 5% dialyzed calf serum. Cells were incubated with 1mCi/ml [^{32}P]orthophosphate (ICN, Irvine, CA) in minus phosphate DMEM containing 5% dialyzed calf serum. Labeling was for 6 to 10 hours.

Preparation of Antiserum against the M_r = 34,000 Protein. The M_r = 34,000 protein was purified according to Erikson and Erikson (2). A New Zealand white rabbit was injected with 100 μg of purified protein at 2 week intervals and bled 5 days after the third injection.

Immunoprecipitation. Labeled cells were immunoprecipitated according to Karess et al. (5) except that the RIPA buffer contained 10 mM EDTA instead of 1 mM EDTA. 4 μl of antiserum was used for each sample. When cells were dissolved in 2% SDS, boiled for 5 min. and the extracts diluted with RIPA buffer lacking SDS prior to immunoprecipitation similar results were obtained.

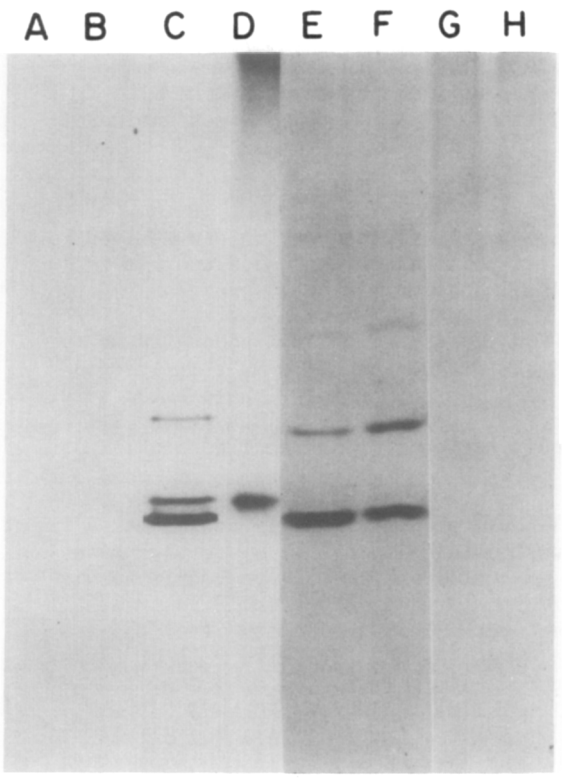
Phosphoamino Acid Analysis. For whole cell analysis, nearly confluent RAT-1 cultures in 35 mm dishes were washed twice with 1 ml minus phosphate DMEM, then 1 ml of minus phosphate DMEM containing 10% dialysed calf serum and 1 mCi [^{32}P] was added. Cells were incubated at 37°C for 12 hours at which time additions were made. After appropriate incubation time, the labeling medium was removed, the monolayers washed twice with 2 ml of Hanks salt solution, and 0.3 ml of lysis buffer (10 mM Tris-HCl, 0.5% SDS, 1 mM EDTA pH 7.4) was added. 0.01 ml of 1 mg/ml protease-free RNAse A (Sigma, St. Louis, MO), 0.5 mg/ml DNAse I (Worthington, Bedford, MA), 0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 7.4 was added and the lysates kept at room temperature for 30 min. Cold trichloracetic acid was added to 20% and samples placed on ice for 30 min. The precipitate was collected by centrifugation, washed twice with 1.0 ml of ice cold 1:1 ethanol:ethyl ether, and dissolved in 6 N HCl. The solutions were placed in glass tubes, sealed under vacuum and heated at 110°C for 90 min. The hydrolysates were lyophilized and analyzed in 2 dimensions. In the first dimension samples were spotted on cellulose thin layer plates (0.1 mm) and electrophoresis was performed at pH 1.9 for 2 hours at 500 V in acetic acid/formic acid/H_2O (78:25:897). Plates were dried and subjected to ascending chromatography in n-butanol/formic acid/iso-propanol/H_2O (3:1:1:1) (6). Analysis of phosphoamino acids in protein bands from SDS-polyacrylamide gels was done according to Decker (7) except the final hydrolysates were analyzed in the two-dimensional system described above.

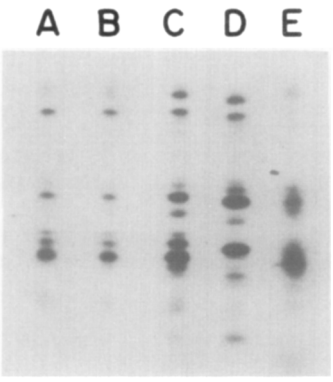
Membrane Preparation and in vitro Phosphorylation. Membranes from RAT-1 cells were prepared according to Thom et al. (8). EGF stimulated phosphorylation of membrane proteins was performed as King et al. (9).

Miscellaneous Methods. Partial V8 protease hydrolysis during electrophoresis was performed as described by Cleveland et al. (10). Two dimensional tryptic maps were prepared according to Beemon and Hunter (11) except that electrophoresis in the first dimension was performed at pH 3.7 (acetic acid:pyridine:H_2O, 100:10:890). EGF binding assays were performed

as Todaro et al. (12) and protein was determined according to Bradford (13). SDS polyacrylamide gels were prepared using the system of Laemmli (14).

Materials. Receptor grade and culture grade EGF were from Collaborative Research (Waltham, MA).





(1) (2)

Fig. 1. Autoradiograms of immunoprecipitates from [^{35}S]methionine labeled and [^{32}P]orthophosphate labeled cells. Procedures were performed as described in "Materials and Methods." Immunoprecipitates from [^{35}S]methionine labeled (A) and [^{32}P]labeled (B) SRA-3Y1 cells using non-immune serum. Immunoprecipitates from [^{35}S]labeled (C) and [^{32}P]labeled (D) SRA-3Y1 extracts using antiserum against the M_r = 34,000 chick cell protein. Immunoprecipitates from [^{35}S]methionine labeled RAT-1 cells in the absence (E) and presence (F) of 100 ng/ml EGF and from [^{32}P]labeled RAT-1 cells in the absence (G) and presence (H) of EGF using antiserum against the M_r = 34,000 chick cell protein. Cells were incubated with EGF for 2 hours prior to immunoprecipitation (F, H).

Fig. 2. Partial V8 protease hydrolysis of the M_r = 35,000 and M_r = 38,000 proteins immunoprecipitated from SRA-3Y1 and RAT-1 cells. Cleavage products derived from the SRA-3Y1 [^{35}S]methionine labeled and SRA-3Y1 [^{32}P]labeled 38,000 protein (A, E), the SRA-3Y1 M_r = 35,000 [^{35}S]methionine labeled protein (B), the [^{35}S]methionine labeled RAT-1 M_r = 35,000 protein (C) and the [^{35}S]methionine labeled chick embryo fibroblast M_r = 34,000 protein (D) are shown. 10 ng of V8 protease was used for each sample.

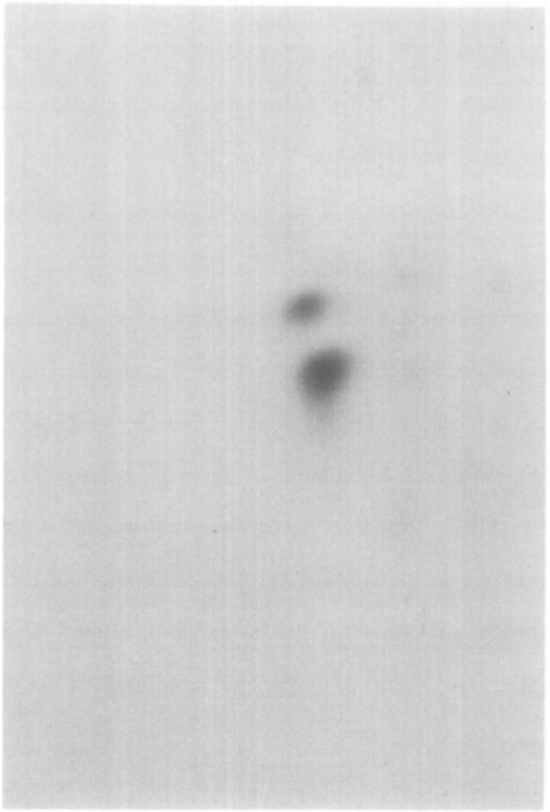


Fig. 3. [^{32}P] containing tryptic peptides from the SRA-3Y1 M_r = 38,000 phosphoprotein. Electrophoresis in the first dimension was from right (+) to left (-). Chromatography was from bottom to top.

Results

Antiserum raised against an M_r = 34,000 chick cell protein purified according to Erikson and Erikson (2) specifically immunoprecipitated two protein bands from [^{35}S]methionine labeled Rous sarcoma virus-transformed 3Y1 rat cells (Fig. 1C). The molecular weights of the upper and lower bands were 38,000 and 35,000 daltons respectively. Immunoprecipitates from [^{32}P]labeled transformed cells showed a single phosphorylated band with the same mobility in SDS-polyacrylamide gels as the M_r = 38,000 [^{35}S]methionine labeled protein (Fig. 1D). Immunoprecipitates from RR 1022 rat cells showed the same two bands (not shown). As previously reported (2), a single [^{35}S]methionine-labeled band was immunoprecipitated from Rous sarcoma transformed chick embryo fibroblasts.

Partial V8 protease hydrolysis of the M_r = 35,000 and 38,000 [^{35}S]methionine labeled bands produced identical peptides (Fig. 2A and B) and peptides generated by V8 treatment of the [^{32}P]labeled 38,000 band co-migrated with [^{35}S]methionine labeled peptides (Fig. 2E).

These results indicate that the M_r = 35,000 and 38,000 proteins are closely related and that the [^{35}S]methionine labeled 38,000 and the [^{32}P]labeled 38,000 protein are probably the same. It is likely that the shift in apparent molecular weight of the protein seen in SDS polyacrylamide gels is due to phosphorylation. If so, the percentage of the protein in the phosphorylated form can be assessed from [^{35}S]methionine counts in the upper and lower bands. Based on this conclusion, 40 - 50% of the protein is phosphorylated in Rous sarcoma transformed rat cells. The phosphate is exclusively on tyrosine residues (not shown) and 2 major phosphotyrosine containing tryptic peptides are present in the M_r = 38,000 band (Fig. 3).

Having established the relationship of these proteins the effect of EGF treatment of normal rat fibroblasts on phosphorylation of the protein was studied. The RAT-1 line was chosen since EGF has been shown to have unusual effects on the cells. EGF is a strong mitogen for these cells stimulating growth past confluence, producing morphological changes, and most strikingly, inducing growth in soft agar (data not shown). However, as seen in Fig. 1, only the M_r = 35,000 band was immunoprecipitated from [^{35}S]methionine labeled control and EGF-treated RAT-1 cells and no increase in phosphorylation was detected in immunoprecipitates from [^{32}P]labeled cells. Similar results were found for NRK clone 49F cells and for 3Y1 cells. No change in phosphorylation was found following incubation with EGF for up to 15 hours. Prolonged exposure of the [^{35}S]methionine labeled RAT-1 immunoprecipitates revealed traces (less than 2% of total 35,000 and 38,000 protein) of an M_r = 38,000 band present in similar amounts in control and EGF-treated cells. EGF was found to stimulate the *in vitro* phosphorylation of tyrosine residues on a M_r = 180,000 protein in membrane

Table 1. Relative levels of acid-stable phosphoamino acids in control and EGF-treated RAT-1 cells.

	Percentage of total recovered phosphoamino acids		
	Phospho-Tyrosine	Phospho-Threonine	Phospho-Serine
RAT-1 (Control)	0.094	7.45	92.46
RAT-1 (50 ng/ml EGF)	0.099	7.70	92.20
Chick embryo fibroblasts	0.053	8.76	91.19
Schmidt-Ruppin A-transformed chick embryo fibroblasts	0.305	8.98	90.72

Cells were [^{32}P]labeled, extracted, protein hydrolysed and analysed in two dimensions as described in the "Materials and Methods". For these data about 2-4 x 10^6 cpm were applied to each thin layer plate. Approximately 15% of the total counts applied were recovered as phospho-amino acids. The phospho-amino acids were located using ninhydrin stained non-radioactive standards, spots were scraped from the plates and radioactivity counted by liquid scintillation counting. Incubation with EGF was for 60 min.

preparations from these cells. Immunoprecipitates from control and EGF-treated A431 epidermoid carcinoma cells revealed an EGF-induced increase in the phosphorylation of the M_r = 38,000 band corresponding to 2 - 5% of the total protein in the two bands (not shown).

As shown in Table 1, EGF treatment did not increase whole cell levels of phosphotyrosine in RAT-1 cells.

Discussion

Protein phosphorylation may play an important role in the mechanism of action of growth promoting peptides such as EGF (3, 4, 6), platelet growth factor (15) and insulin (16). Loss of growth control induced by a number of tumor viruses also appears to involve protein phosphorylation (17). The M_r = 34,000 protein first described by Radke and Martin (1) is of particular interest since increased phosphorylation of this protein occurs after transformation by several avian sarcoma viruses (18) and also occurs in the A431 epidermoid carcinoma cell line (which possesses an unusually high number of EGF receptors) after administration of EGF (3, 4).

Unfortunately, EGF is not a mitogen for this cell line, but instead inhibits growth (19) perhaps by activating the over-produced receptor-kinase system to somewhat indiscriminately phosphorylate proteins.

It seemed that to more accurately determine the role of phosphorylation of the 34,000 protein in the EGF system normal cell lines for which EGF stimulates cell proliferation should be studied. The RAT-1 line was chosen since it possessed about 25,000 EGF receptors per cell (a relatively typical number) and since EGF treatment of these cells results in a strong proliferative response.

The results of this study indicate that the M_r = 34,000 protein from RAT-1 cells and from two other normal rat fibroblast cell lines is not phosphorylated in response to EGF treatment. These data suggest that increased phosphorylation of this protein is not necessary for EGF to exert its growth stimulatory effects.

In addition, it was found that the phosphorylated form of this protein in the two mammalian cell types examined (rat and human) undergoes a shift in mobility in SDS-polyacrylamide gels from an apparent molecular weight of 35,000 to M_r=38,000. This finding was useful in estimating the percentage of the protein which is in the phosphorylated form. For the two Rous sarcoma transformed rat cell lines (the SRA-3Y1 and RR 1022 lines) 40 - 50% of the protein was phosphorylated which is 3 to 4 fold higher than the percentage reported for Rous sarcoma transformed chick cells (20). In addition, the phosphorylated protein from the transformed rat cells contained only phosphotyrosine; no phosphoserine was found as in the chick cell protein (12).

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References

1. Radke, K., and Martin, G.S. (1979) Proc. Nat. Acad. Sci. 76, 5212-5216.
2. Erikson, R.L., and Erikson, E. (1980) Cell 21, 829-836.
3. Hunter, T. and Cooper, J.A. (1980) Cell 24, 741-752.
4. Erikson, E., Shealy, D.J., and Erikson, R.L. (1981) J. Biol. Chem. 256, 11381-11384.
5. Karess, R.E., Hayward, W.S., and Hanafusa, H. (1980) Proc. Nat. Acad. Sci. 76, 3154-3158.
6. Ushiro, H., and Cohen, S. (1980) J. Biol. Chem. 255, 8363-8365.
7. Decker, S. (1981) Proc. Nat. Acad. Sci. 78, 4112-4115.
8. Thom, D., Powell, A.S., Lloyd, C.W. and Rees, D.A., Biochem. J. 168, 187-194.
9. Cohen, S., Carpenter, G., and King, L. (1980), J. Biol. Chem. 255, 4834-4842.
10. Cleveland, D. W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102-1106.
11. Beemon, K., and Hunter, T., (1978) J. Virol. 28, 551-566.
12. Todaro, G.J., DeLarco, J.E., and Cohen, S. (1976) Nature 264, 26-30.
13. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
14. Laemmli, U.K. (1970) Nature 227, 680-685.
15. Ek, B., Westermark, B., Wasteson, A., and Heldin, C. (1982) Nature 295, 419-420.
16. Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., and Kahn, C.R. (1982) Nature 298, 667-669.
17. Hunter, T., Sefton, B.M., and Cooper, J.A. (1981) Protein Phosphorylation: Cold Spring Harbor Conferences on Cell Proliferation VIII, pp. 1189-1202, Cold Spring Harbor Laboratory, New York.
18. Erikson, E., Cook, R., Miller, G.J., and Erikson, R.L. (1981) Molec. and Cell Biol. 1, 43-50.
19. Gill, G. N., and Lazar, C.S. (1981) Nature 293, 305-307.
20. Radke, K., Gilmore, T., and Martin, G.S. (1980) Cell 21, 821-828.