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SERINE PHOSPHORYLATION OF THE v-rel ONCOGENE PRODUCT/pp40 COMPLEX

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The transforming protein encoded by the v-rel oncogene of reticuloendotheliosis virus has been purified from REV-T transformed lymphoid cells by sequential DEAE-Sepharose and immunoaffinity chromatography. The purified preparation consisted of pp59$^{v\text{-}rel}$ and the 40 kDa cellular protein which is complexed with the v-rel oncogene product in transformed cells as well as some minor proteins. Incubation of this purified preparation in the presence of Mg^{2+} and (γ-^{32}P)ATP resulted in phosphorylation of both pp59$^{v\text{-}rel}$ and the 40 kDa protein. This preparation was also able to phosphorylate casein on serine residues. Immunoprecipitates obtained from extracts of REV-T transformed lymphoid cells labeled with ^{32}P-orthophosphate contained 59 and 40 kDa phosphoproteins. Both pp59$^{v\text{-}rel}$ and the 40 kDa protein were phosphorylated on serine residues in transformed cells.

Reticuloendotheliosis virus (REV-T) is an extremely virulent retrovirus which induces an invariably fatal lymphoma in avian species within 7-10 days (1). REV-T transforms and prevents the further differentiation of very immature avian lymphoid cells (2,3,4,5). REV-T contains an oncogene, termed v-rel, whose expression converts a normal cell to a tumorigenic state (2). The v-rel oncogene and its product are distinct from other transforming proteins (6,7,8). The protein encoded by v-rel has recently been identified in transformed cells as a phosphoprotein with an apparent molecular mass of 55-59kDa, designated pp59$^{v\text{-}rel}$ (9,10,11,12). This protein is synthesized in very low amounts constituting between 0.003 - 0.004% of the metabolically labeled proteins and has a half life of 6-8 hrs in transformed lymphoid cells (13). The v-rel protein is located principally in the cytosolic fraction of

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transformed lymphoid cells (10,13,14) and is complexed with a 40 kDa cellular phosphoprotein to form a 400 kDa heteropolymer (15).

The role of pp59$^{v\text{-}rel}$ in cell transformation is unknown. Immune complexes recovered from extracts of REV-T transformed cells with antisera directed against v-rel bacterial fusion proteins or with antisera against v-rel related synthetic peptides display protein kinase activity (11,13). During these *in vitro* kinase reactions, pp59$^{v\text{-}rel}$ becomes phosphorylated. In this manuscript we report that when pp59$^{v\text{-}rel}$ was purified by chromatography on DEAE-Sepharose and a monoclonal antibody affinity column, the highly purified pp59$^{v\text{-}rel}$/40 kDa complex still contains a serine kinase activity. Furthermore, both pp59$^{v\text{-}rel}$ and the 40 kDa cellular protein complexed to the v-rel protein were phosphorylated on serine residues in transformed lymphoid cells.

MATERIALS AND METHODS

DEAE-Sepharose CL-6B, CNBr-activated Sepharose 4B and Protein A-Sepharose were purchased from Pharmacia Inc (Piscataway, NJ). Dephosphorylated and autoclaved casein, nonimmune IgG-agarose, Protein-A agarose and phosphoamino acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). Goat antimouse IgG and goat antirabbit IgG conjugated to alkaline phosphatase were from BioRad (Richmond, CA). [γ^{32}-P]ATP was purchased from New England Nuclear (Boston, MA).

A mouse monoclonal antibody directed against the product of the 3' end of the v-rel oncogene product has been produced. This monoclonal antibody recognizes the full size v-rel oncogene product, pp59$^{v\text{-}rel}$, in REV-T transformed cells. Details of production and characterization of the monoclonal antibody has been described elsewhere (15). The production of rabbit polyclonal antibodies directed against the amino end, the middle portion and the carboxy end of the v-rel oncogene product synthesized in *E. coli* as tryp-E-v-rel fusion proteins has been previously described (9,13).

The mouse monoclonal antibody was purified by affinity chromatography on a protein-A agarose column. Following elution, it was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instruction (Pharmacia Inc.).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (16).

Western immunoblotting was performed as follows: Proteins were separated by SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose. Proteins were visualized on nitrocellulose by using monoclonal or polyclonal antibodies as the primary antibodies and alkaline phosphatase conjugated to either goat anti-mouse IgG or goat anti-rabbit IgG as the secondary reagent. Staining was by incubation in P-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate in 50 mM sodium bicarbonate pH 9.8, 100 mM NaCl and 5 mM $MgCl_2$.

Isolation of the v-rel oncogene product: A nonvirus-producing REV-T transformed cell line (REC4#1) (15) was used as a source of the v-rel oncogene product. Exponentially growing cells in RPMI 1640 medium, supplemented with 5% fetal bovine serum, were collected by centrifugation at

3000 rpm for 10 min in a Beckman JA-10, washed once with phosphate buffered saline (PBS) and homogenized with a polytron unit in 100 ml of 25 mM Tris-Cl pH 7.5, 2 mM EDTA, 10 mM EGTA, 0.2 mM PMSF, 1 mM benzamidine and 0.1% (v/v) 2-mercaptoethanol. The cell extract was examined microscopically to ensure that at least 70% of the cells were disrupted. Cytosolic extracts of REV-T transformed cell lines were centrifuged at 45000 rpm in a Beckman Ti 50-2 rotor for 30 min. The supernatant fraction was collected, diluted 2-fold with 25 mM Tris-Cl pH 7.5, 0.2 mM EGTA, 0.2 mM PMSF, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethnaol (buffer A) and loaded onto a DEAE-Sepharose CL-6B column (2.5 x 10 cm) equilibrated in buffer A. The column was washed extensively with buffer A + 25 mM NaCl and then stripped with buffer A + 300 mM NaCl. The eluted proteins were diluted 2-fold with buffer A, passed through a column of nonimmune mouse IgG-Agarose (1.5x5cm) and mixed with 5 ml of monoclonal antibody bound to Sepharose-4B and equilibrated in buffer A + 0.150 mM NaCl. The mixture was rotated end to end for 2 hr at 4°C and then loaded onto a column. The column was washed with buffer A + 1 M NaCl, followed by buffer A + 0.5 M NaCl followed by buffer A only until no protein was eluted from the column. The column was then washed with 10 mM sodium phosphate pH 6.0 containing 0.05% (v/v) 2-mercaptoethanol and stripped with 10 mM sodium phosphate pH 6.0, 0.05% (v/v) 2-mercaptoethanol and 1.2 M $MgCl_2$. The eluted v-rel oncogene product-preparation was dialyzed against 50 mM Tris-Cl pH 7.5, 0.1 mM EGTA, 0.2 mM PMSF, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol and 10% glycerol. Twenty to 30 μg of highly purified v-rel oncogene preparation was obtained by this procedure.

Protein was determined by the method of Bradford (17) using bovine serum albumin ($Abs^{1\%}_{280\ nm}$ = 6.5) as standard.

Phosphoamino acid analysis: ^{32}P-labeled proteins were hydrolyzed in 6 M HCl for 2 hr at 110°C in an evacuated tube and the hydrolysate was subjected to high voltage electrophoresis at pH 3.5 on Whatman 3 MM paper. The buffer consisted of pyridine, acetic acid and water (1:10:189). ^{32}P-labeled phosphoamino acids were located by autoradiography and phosphoamino acid standards were located by ninhydrin staining.

RESULTS AND DISCUSSION

Proteins in cytosolic extracts of an REV-T transformed lymphoid cell line were separated on sequential DEAE-Sepharose CL-6B (Pharmacia, Inc.) and immunoaffinity chromatography employing a MoAb against $pp59^{v\text{-}rel}$ (Fig. 1, Panel A). The proteins present in the cytosolic extract (lane 1), those which were concentrated by DEAE-Sepharose chromatography (lane 2) and eluted from the immunoaffinity column (lane 3), were separated by SDS-PAGE and detected by silver staining. Following immunoaffinity purification, two major proteins with a molecular mass of 59 and 40 kDa as well as some minor proteins were detected. The 59 kDa protein was recognized by MoAb directed against the product of the 3' end of the v-rel oncogene synthesized in E. coli as a trpE/v-rel fusion protein (15) (Fig. 1, Panel B). The 59 kDa protein also reacted with immunoglobulins obtained from polyclonal rabbit

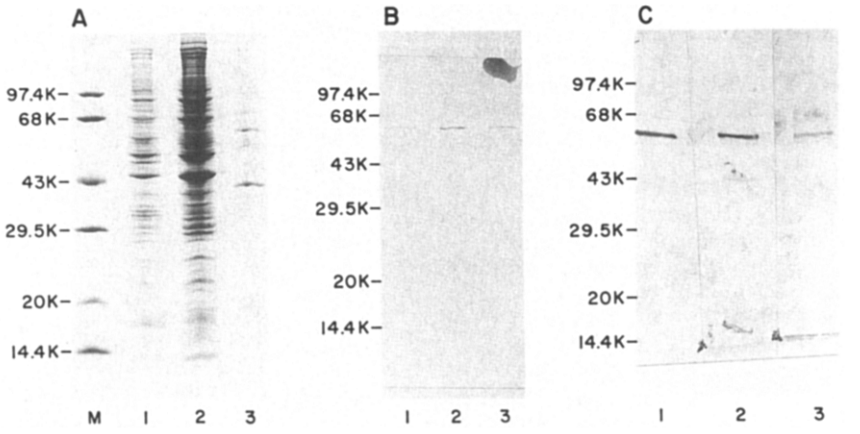

Fig. 1. Isolation and purification of the v-rel oncogene product from transformed lymphoid cells.
Panel A represents proteins in extracts of REV-T transformed lymphoid cells (lane 1); proteins eluted from the DEAE-Sepharose (lane 2) and proteins from sequential chromatography on DEAE-Sepharose and MoAb affinity column (lane 3). Lane M are marker proteins, phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29.5 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa).
Panel B represents the detection of pp59$^{v\text{-}rel}$ by Western immunoblotting using MoAb directed against the carboxyl-terminal region of pp59$^{v\text{-}rel}$ in extracts of REV-T transformed lymphoid cells (lane 1); from chromatography on DEAE-Sepharose (lane 2) and from sequential chromatography on DEAE-Sepharose and MoAb used in panel A monoclonal antibody affinity column (lane 3).
Panel C represents the detection of pp59$^{v\text{-}rel}$ by Western immunoblotting, using polyclonal antisera directed against the amino-terminal region (lane 1), the middle region (lane 2) and the carboxyl-terminal region (lane 3) of pp59$^{v\text{-}rel}$.

antisera directed against the amino-terminal, middle, and carboxy-terminus of the v-rel oncogene product (13) (Panel C).

The pp59$^{v\text{-}rel}$/40 kDa complex eluted from the immunoaffinity column underwent phosphorylation when it was incubated with 10 mM $MgCl_2$ and [γ^{32}-P]ATP. Both the 59 kDa and the 40 kDa proteins were phosphorylated although the phosphorylation of the 40 kDa protein was far more extensive than the 59 kDa protein (Fig. 2, Panel A, lane 1). The 40 kDa protein and pp59$^{v\text{-}rel}$ purified by sequential Sephacryl S-200 and immunoaffinity chromatography were also phosphorylated *in vitro* (15) although the extent of phosphorylation of the 40 kDa protein was far less. The use of DEAE-Sepharose rather than Sephacryl chromatography is more effective in preserving the kinase activity associated with the pp59$^{v\text{-}rel}$/40 kDa complex perhaps because of the reduced length of time required to purify the complex. The incorporated phosphate in

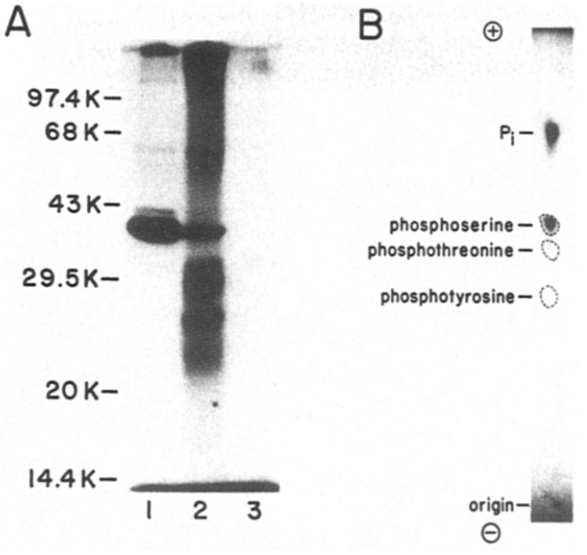

Fig 2. Phosphorylation of the highly purified preparation containing the v-rel oncogene product. The purified preparation from MoAb column was incubated at 30°C for 10 min in 50 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol; 10 mM $MgCl_2$ and 0.1 mM [γ^{32}-P]ATP. Panel A. Lane 1 contains the v-rel oncogene product preparation only; Lane 2 contains the v-rel oncogene product preparation and 40 μg casein; Lane 3 contains 40 μg casein. Reaction was stopped by heating the incubation mixtures for 5 min at 100°C with an equal volume of denaturing sample buffer and the mixture was subjected to SDS-polyacrylamide gel electrophoresis. The gel was washed extensively, dried and exposed to x-ray film. Bars indicate the position of marker proteins described in Figure 1.
Panel B. Phosphoamino acid analysis of the ^{32}P-labeled protein from the phosphorylation reaction containing casein and highly purified preparation of v-rel oncogene product. A sample of ^{32}P-labeled proteins was hydrolyzed and subjected to high voltage paper electrophoresis.

the 40 kDa protein was labile to treatment with 1 M potassium hydroxide at 55°C, suggesting the absence of phosphotyrosine. The highly purified pp59$^{v\text{-}rel}$/40 kDa complex was also able to phosphorylate casein (Fig. 2, Panel A, lanes 2 and 3) on serine residues (Fig. 2, Panel B). The multiple phosphorylated polypeptides (Fig. 1, Panel A, lane 2) represents heterogeneity of the casein preparation.

To define whether pp59$^{v\text{-}rel}$ and the 40 kDa cellular phosphoprotein are also phosphorylated on serine residues *in vivo*, the complex was immunoprecipitated with the MoAb against pp59$^{v\text{-}rel}$ from extracts of ^{32}P-orthophosphate labeled cells (Fig. 3, Panel A, lane 2). The polypeptides were separated by PAGE, eluted, hydrolyzed and the phosphoamino acid identified by high voltage paper electrophoresis. As indicated in Fig. 3,

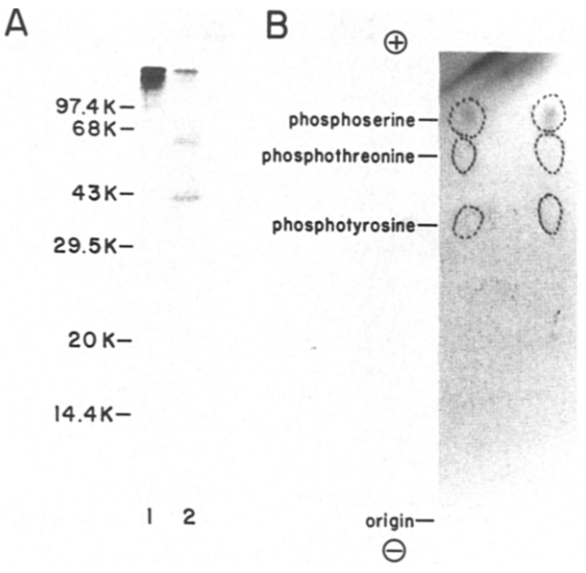

Fig. 3. Immunoprecipitation of phosphoprotein from REV-T transformed cells with MoAb directed against pp59$^{v\text{-}rel}$. REV-T transformed lymphoid cells were grown in the presence of ^{32}P-orthophosphate and extracts were immunoprecipitated with MoAb directed against v-rel oncogene product in an immunoprecipitation buffer consisting of 20 mM Tris-Cl pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% (v/v) Triton x-100, 0.05% (v/v) sodium dodecyl sulfate, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM benzamidine and 0.2 mM PMSF. The cell lysate was clarified by centrifugation and 2-5 μg of affinity purified MoAb was added per ml of cell lysate (200-500 μg total protein). Following incubation for 1 hr at 4°C, protein A-Sepharose (200 μl) was then added and the incubation continued for 1 hr at 4°C. Immune complex was collected by centrifugation, washed three times with immunoprecipitation buffer, resuspended in SDS-containing sample buffer, boiled for 3 min at 100°C and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.
Panel A. Lane 1 contains phosphoprotein precipitated with preimmune serum and Lane 2 contains phosphoproteins precipitated with monoclonal antibody directed against pp59$^{v\text{-}rel}$.
Panel B. Phosphoamino acid analysis of pp59$^{v\text{-}rel}$ (lane 1) and pp40 (lane 2). Immunoprecipitations were carried out as described above. The proteins were eluted from the SDS-PAGE and subjected to acid hydrolysis and high voltage paper electrophoresis.

Panel B, both pp59$^{v\text{-}rel}$ and pp40 were phosphorylated on serine residues in REV-T transformed lymphoid cells.

The pp59$^{v\text{-}rel}$/40 kDa complex purified from REV-T transformed cells by sequential DEAE-Sepharose and immunoaffinity chromatography was associated with an *in vitro* serine protein kinase activity. The kinase present in these preparations phosphorylated pp59$^{v\text{-}rel}$, the 40 kDa protein and casein on serine residues. Likewise, both pp59$^{v\text{-}rel}$ and the 40 kDa cellular protein immunoprecipitated from cytosolic extracts of REV-T transformed cells were phosphorylated on serine residues. At the present time, it is unclear

whether the kinase activity is intrinsic to pp59$^{v\text{-}rel}$ or to the 40 kDa protein which is complexed with v-rel in transformed cells. Antisera is currently being generated against the 40 kDa protein in an effort to define whether the 40 kDa cellular protein associated with pp59$^{v\text{-}rel}$ has serine kinase activity. Two arguments would suggest that the v-rel oncogene product, pp59$^{v\text{-}rel}$ is not a protein kinase. First, the nucleotide sequence of v-rel and its predicted amino acid sequence (7) are distinct from the transforming sequence of those oncogenes which encode either tyrosine (18) or serine/threonine protein kinase (19,20,21). The v-rel gene does not contain the 30 kDa catalytic domain of known tyrosine or serine/threonine cellular kinase (22). Second, the v-rel nucleotide sequence does not contain the consensus sequence of an ATP-binding domain which is found in tyrosine and serine/threonine protein kinases (23). However, the possibility that the v-rel oncogene product may represent a novel form of protein kinase cannot be ruled out entirely. There is now evidence which supports the existence of a protein kinase which does not possess any of the sequences typically defined as the catalytic domain of the tyrosine or serine/threonine protein kinase family. This protein kinase is termed NR_{11} and is responsible for the phosphorylation and dephosphorylation of another protein termed NR_1 (24,25).

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