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Protein Kinase Activity of Polysome-Ribosome Preparations from Poliovirus Infected Cells

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SUMMARY. Polysome-ribosome preparations from poliovirus infected HeLa cells contain 10-15 fold more protein kinase activity in vitro than similar preparations from uninfected cells. Five peptides of mol. wt. 135K, 85K, 65K, 28K and 14K are phosphorylated by polysome-ribosome preparations from infected cells. Extracts obtained from cells infected in the presence of guanidine phosphorylate the same proteins whereas polysome-ribosome extracts obtained from uninfected cells treated with cycloheximide phosphorylate the 135K peptide and two others with mol. wt. values of 77K and 60K. Over 90% of the 135K peptide and over 70% of the other peptides can be washed off ribosomes with 0.5 M KCl.

Picornaviruses such as poliovirus inhibit the synthesis of cellular proteins soon after infection of a variety of cells such as HeLa, monkey kidney, human diploid (HEL), Human epithelial (WISH) and others (1, 2). In poliovirus infected cells that are co-infected with vesicular stomatitis virus (3, 4) or herpesvirus (5) only poliovirus mRNA is translated. The blockage of cellular protein synthesis requires a functional viral genome since UV inactivated virus fails to inhibit cellular protein synthesis (6). The mechanism for shutoff of host protein synthesis has not been elucidated. A recent hypothesis attributing shutoff of host protein synthesis to an increase in cellular monovalent cation concentration is attractive (7, 8). However, mengovirus, another picornavirus, does not alter the intracellular monovalent cation concentration during the period of time cellular protein synthesis declines (9).

Globin synthesis in rabbit reticulocytes is regulated by a protein kinase that phosphorylates peptides essential for initiation of protein synthesis (10, 11, 12). Phosphorylation blocks the initiation of globin synthesis. Also SV40 transformed African green monkey kidney cells have the ability to translate mRNA of adenovirus whereas untransformed cells fail to translate some species of late adenovirus mRNA (13). Transformed cells contain a ribosome-bound phosphoprotein that is absent in untransformed

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cells (14) and it is possible that the translational activity of ribosomes has become functionally changed by phosphorylation. This report shows that poliovirus infection enhances protein kinase activity of polysome-ribosome preparations assayed in vitro.

Materials and Methods

HeLa-0 cells were obtained from Flow Laboratories (Rockville, MD) and propagated with Earle's-MEM medium containing 8% newborn calf serum, 50 µg/ml streptomycin and 50 units/ml penicillin. Cells were grown in Corning 490 cm^2 roller vessels to an average of 1.5 x 10^8 cells per roller. Infection was carried out with 100 pfu/cell with the LSc strain of type 1 poliovirus as described (15).

Polysome-ribosome preparations for measuring protein kinase activity were obtained from infected and mock-infected cells 3 hr post-infection at 35C. Cells were infected or mock-infected on monolayer for 1 hr, and detached from the vessels with 0.25% trypsin - 15 mM EDTA. Usually 8 x 10^8 cells were collected in 200 ml cold medium, sedimented at 100 g, 10 min 4C and resuspended to 5 x 10^6 cells/ml in warm medium. After 2 more hr at 35C on a shaker cells were pelleted and washed 3X with 100 ml portions of Hank's balanced salt solution (16) and resuspended in two volumes of polysome extract buffer (50 mM Tris·HCl pH 7.4-100 mM KCl - 7 mM $Mg(OAc)_2$ - 1 mM dithiothreitol (DDT) - 0.25% Nonidet P40). After lysing cells with 10 strokes in a Dounce homogenizer nuclei were removed by sedimentation at 200 g, 10 min, 4C and mitochondria were sedimented at 10,000 g, 10 min 4C in a Spinco SW56 rotor. The supernates were layered over 0.3 ml 15% sucrose (W/W) in polysome extract buffer and centrifuged 3½ hr, 250,000 g, 4C in a Spinco SW56 rotor. The surfaces of the polysome-ribosome pellets were rinsed with assay buffer (50 mM Tris·HCl pH 7.4-35 mM KCl-10 mM Mg $(OAc)_2$ - 1 mM DDT) and the pellets were resuspended to 8-10 mg/ml protein with a homogenizer. Protein assays were performed with the standard Folin & Ciocalteau reagent with a protein standard containing 5% human albumin and 3% human gamma globulin (Sigma). Volumes of 0.2 ml of extract were stored in provials at -86C in a Revco freezer.

To assay kinase activity in vitro polysome-ribosome extracts were combined with an equal volume of assay buffer lacking $Mg(OAc)_2$ but containing 15 µCi $\gamma[^{32}P]$-ATP (100-3000 Ci/mM, NEN) so that final reaction mixtures of 0.3 ml contained 50 mM Tris·pH 7.4-35 mM KCl - 5 mM Mg $(OAc)_2$ - 1 mM DDT and 15 µCi $\gamma[^{32}P]$-ATP. After 30 min at 32C five volumes of 10% trichloroacetic acid (TCA) were added, the precipitates were collected by centrifugation, washed three times with ether-ethanol (1:1) and once with ether. After drying the proteins were solubilized with 0.4 ml 4% SDS-2 M Urea - 2% 2-mercaptoethanol for 5 min in a boiling water bath. After dialysis against three changes of 2000 volumes of dialysis buffer (10 mM phosphate, pH 7.2 - 0.1% SDS - 1% 2 mercaptoethanol) electrophoresis was performed with 7.5% polyacrylamide disc gels for 19.5 hr at 5 mA/gel as described (15).

To determine whether phosphoproteins can be washed off ribosomes 14 volumes of hypertonic buffer (10 mM Tris·HCl pH 7.4-1.5 m $MgCl_2$ - 500 mM KCl) were added at the end of incubation at 32C and the samples were centrifuged 3½ hr 250,000 g in a Spinco SW56 rotor. About 90% of the supernates were withdrawn and processed as above for polyacrylamide gel electrophoresis. The surfaces of the pellets were washed with hypertonic buffer, the pellets were resuspended in hypertonic buffer and resedimented. The supernates from the final sedimentation were discarded. Pellets were solubilized and examined by gel electrophoresis.

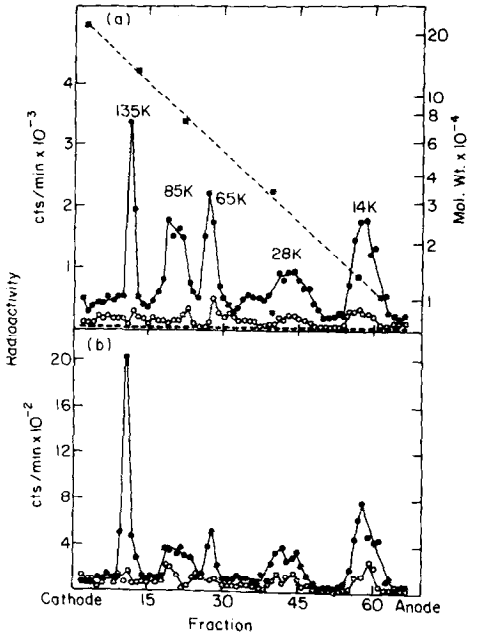

Fig. 1. Polyacrylamide gel analysis of proteins phosphorylated in vitro. (a) Poliovirus infected cells (●), uninfected cells (○), infected cells extracts treated with 200 µg/ml pronase for 30 min at 35C at the end of in vitro assay (---). (b) Supernate of the 0.5 M KCl wash from extracts of infected cells phosphorylated in vitro (●), Ribosomal pellet after KCl wash (○). Mol. wt. standards are bovine serum albumin trimer 204K, monomer 68K, β-galactosidase 130K, pepsin 35.5K, and β-chymotrypsin 14K.

Results

Extracts of polysomes-ribosomes from poliovirus infected HeLa cells generally incorporate 10-15 fold more γ[^{32}P] from ATP into proteins than preparations from uninfected cells. Control studies show that incorporation occurs into proteins and not nucleic acids or phospholipids. Treatment with 200 µg/ml pronase solubilizes 97% of the incorporated ^{32}P and 100 µg/ml hog intestinal alkaline phosphatase solubilizes 87% of the ^{32}P after 30 min 35C. After 1 hr 95C in 10% TCA all radioactivity remained in the precipitates. Phosphate esters are hydrolyzed by alkaline phosphatase (17) and the failure to completely render radioactivity acid soluble with the esterase might merely reflect inaccessibility of substrate sites.

Polyacrylamide gel electrophoresis of phosphorylated proteins consistently showed 5 peptides in three different preparations. Fig. 1a presents the results from one such extract where the mol. wt. values of peptides are

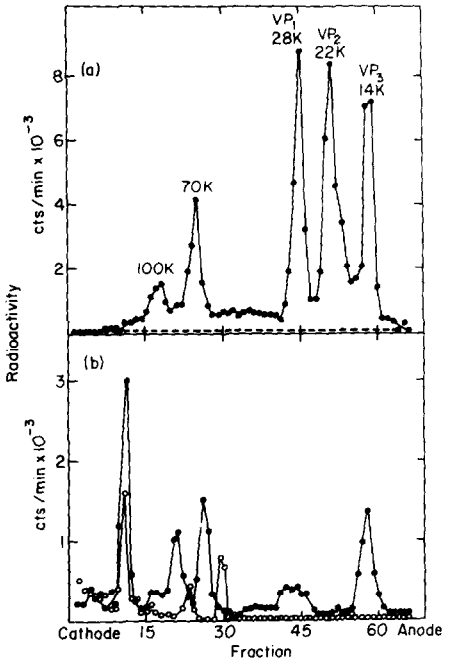

Fig. 2. Polyacrylamide gel analysis of viral peptides produced *in vivo* and peptides phosphorylated *in vitro* by extracts from cells treated with guanidine or cycloheximide. (a) Viral peptides produced *in vivo* in the absence (●) and presence (---) of 2 mM guanidine. Cells were infected with poliovirus and labeled 2 hr 45 min to 3 hr 45 min after infection with 2 μCi/ml ^{14}C-amino acids as described (15). The guanidine treated culture contained 2 mM guanidine throughout infection. (b) Ribosome-Polysome extracts were prepared from infected cells treated with 2 mM guanidine throughout infection (●) and uninfected cells treated with 11 μg/ml cycloheximide for 90 min (○). Proteins phosphorylated by the standard *in vitro* reaction mixture were analyzed.

designated in the figure. In control preparations two proteins migrating slightly faster than the 85K, and 65K peptides found in extracts of infected cells is noted. They consistently migrated according to mol. wt. values of 77K and 60K.

Peptides involved in the initiation of protein synthesis are not stable components of ribosomes and can be washed off ribosomes with 0.5 M KCl (10, 11, 12). After incubation of extracts from virus infected cells with $\gamma[^{32}P]$-ATP the preparations were diluted with hypertonic buffer containing 0.5 M KCl and processed as outlined in Materials and Methods. Analysis of washed ribosomes and the wash fluid by polyacrylamide gel electrophoresis shows that over 90% of the 135K phosphoprotein and at least 70% of the other

phosphoproteins are removed with hypertonic buffer (Fig. 1b). This is probably an underestimate because the second wash fluids were discarded. However, even after two washes with 0.5 M KCl broad peaks of radioactivity migrating with average mol. wt. 85K, 28K and 14K still contain about 30% of the counts in ribosomal pellets and it is possible that structural proteins of ribosomes are also phosphorylated.

The increased protein kinase activity of polysome-ribosome preparations from infected cells might reflect a critical change in translational capabilities due to virus infection. However, changes in phosphorylation capacity could also arise as a secondary effect of blockage of host protein synthesis. To determine whether enhanced phosphorylation of proteins as shown in Fig. 1 is characteristic of poliovirus infection two control studies were performed. First cells were infected with poliovirus in the presence of 2 mM guanidine, a compound that blocks synthesis of detectable viral proteins but permits turnoff of host protein synthesis (18). Secondly uninfected cells were treated with 11 μg/ml cycloheximide and after 90 min were wahsed, polysome-ribosome extracts were prepared and examined for protein kinase activity in vitro. This level of cycloheximide blocks 89% protein synthesis within 30 min.

Part (a) of Fig. 2 shows that in the absence of guanidine only the commonly observed virus specific peptides are produced in vivo (15). In the presence of guanidine no detectable proteins, viral or cellular, are synthesized. This shows that in the presence of guanidine poliovirus completely blocks cellular protein synthesis even though the normal production of virus specific proteins is blocked. The gel profile of proteins phosphorylated by extracts from infected cells exposed to guanidine is identical to that from extracts derived from infected cells in the absence of guanidine (Fig. 2b). Extracts from cycloheximide treated cells phosphorylate the 135K peptide to half the extent noted for infected cells and phosphorylation of the 77K and 60K proteins is increased. It appears that phosphorylation of peptides with mol. wt. values of 85K, 65K, 28K and 14K is induced by poliovirus infection but not by inhibition of cellular protein synthesis with cycloheximide.

Several preliminary studies also show that polysome-ribosome extracts from poliovirus infected Vero cells have 10-15 fold higher protein kinase activity than preparations from uninfected Vero cells.

Discussion

Phosphorylation of proteins is important in the regulation of glycolysis in skeletal muscle (19), lipolysis (20), the activity of pyruvate

dehydrogenase (21), and the initiation of protein synthesis in rabbit reticulocytes (10, 11, 12). HeLa cells infected with vaccinia virus contain two phosphoproteins associated with 40_S ribosomal subunits (22) while mouse L cells infected with vesicular stomatitis virus contain several ribosomal phosphoproteins not found in control cells (23). In the later study phosphorylation of ribosomal proteins was considered to be a consequence rather than a cause of the cytopathic state.

This report shows that polysome-ribosome extracts from poliovirus infected cells have increased protein kinase activity compared to extracts from uninfected cells. The phosphorylation is distinct from that induced by cycloheximide, an inhibitor of protein synthesis. These findings do not prove that phosphorylation of cellular proteins is responsible for dominance of translation by poliovirus but based on studies with reticulocytes are suggestive. In this respect changes in intracellular polyelectrolytes following virus infection as suggested by others (7, 8) might alter protein kinase activity and consequently translational capacity after infection.

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