Glucose binding and transport proteins extracted from fast-growing chicken fibroblasts

(serum/uptake/phosphorylation/purification)

SUNG G. LEE* AND FRITZ LIPMANN†

The Rockefeller University, New York, New York 10021

Contributed by Fritz Lipmann, August 28, 1978

ABSTRACT Preconfluent or confluent fibroblasts grown in 5% serum medium yielded, without cell lysis, all the glucose-binding protein and most of the transport-stimulating activity in the cell wash fluid obtained with a 10 mM sodium octanoate-containing solution. For assay, octanoate was removed, and after the binding protein was labeled with [^{14}C]glucose, the factors were chromatographed on Sephadex G-200 and the transport-stimulating and factor-bound [^{14}C]glucose activities were measured. Three peaks were separated, which more or less overlapped for both functions; upon chromatography on DEAE-cellulose, these peaks yielded overlapping or separate peaks for the two functions, presumably indicating their separability. Serum, when similarly chromatographed, showed only peaks for transport which, with the exception of one major peak with both functions, more or less overlapped with those from the wash fluid. Glucose transport rates, when compared in fibroblasts grown in glucose and in fructose and in Rous sarcoma virus-transformed cells grown in glucose, were in the proportion of 1:3.7:6.3. Addition of extracted transport protein stimulated the transport of both the glucose-grown and fructose-grown normal cells but showed no effect on the transport of transformed cells. Addition of transport protein induced the formation of [^{14}C]deoxyglucose 6-phosphate in amounts proportional to the increased transport of [^{14}C]deoxyglucose into fibroblasts. On sodium dodecyl sulfate electrophoresis, using the tightly bound [^{14}C]glucose for assay, purified binding protein yielded large fractions of 36,000 and 18,000 and small ones of 55,000 and 73,000 daltons; the 18,000-dalton fraction is supposedly the monomeric form of the binding protein.

We recently reported (1) on the isolation of a glucose-binding fraction obtained from a postconfluent culture of normal chicken embryo fibroblasts, most of which was rather loosely bound to the cell membrane, and compared it to the glucose-binding fraction obtained from Rous sarcoma virus (RSV)-transformed chicken fibroblasts. In cells transformed with the temperature-sensitive RSV TS68 of Kawai and Hanafusa (2), most of the binding factor was tightly bound and could only be extracted with Triton X-100. Quantitatively, all extractable binding factor, measured as described (1), was found to be about 2.5 times higher in transformed cells as compared to confluent normal fibroblasts, and the uptake of glucose by the transformed cells was similarly higher than it was by normal ones. Furthermore, using starved cells, addition of the fraction containing the glucose-binding factor stimulated the low uptake of a quiescent cell culture up to 7-fold until a plateau was reached.

This effect compared favorably with well-known stimulatory effects on glucose uptake by serum and insulin (3-8). In contrast to a rather abundant increase in DNA synthesis caused by serum and insulin addition, however, the glucose-binding-transfer fraction did not cause such an increase; i.e., it did not stimulate growth (8).

In the experiments presented in this paper, preconfluent or just confluent secondary fibroblast cultures grown in 5% serum were used. To assay for glucose-binding and transport proteins, we prepared 10 mM octanoate washes; these two functions were obtained, overlapping or separate, by Sephadex G-200 followed by DEAE-cellulose chromatography. Fractions from the second chromatogram were used in a variety of experiments, partly to compare responses of normal and RSV-transformed cells.

METHODS

Large-Scale Preparation of Glucose-Binding-Transport Fraction. The secondary culture of chicken embryo fibroblasts was grown in roller bottles in a medium containing 5% serum, 0.3% tryptose phosphate broth, and Scherer's nutrients. At the time of cell confluency, the culture medium was drained and the cells attached to the bottles were rinsed with 50 ml of glucose-free Hanks' balanced salt solution. After the Hanks' solution was drained, 30 ml of 5 mM triethanolamine-HCl, pH 7.4/100 mM NaCl/2 mM EDTA/10 mM Na octanoate was added to each bottle; the cells were detached by exposing them to the solution for about 30 min by rolling the bottles. The wash fluid and the detached cells were collected and the latter were removed by centrifugation. About 1 liter of the wash fluid was lyophilized to about 40 ml; it was then thawed and a small amount of insoluble material was removed by centrifugation. Lyophilization to complete dryness was avoided because completely dried cell wash material is difficult to solubilize.

The concentrated cell wash fluid was applied to a Sephadex G-50 column equilibrated with 5 mM triethanolamine buffer, pH 7.4/2 mM EDTA/50 mM NaCl/0.02% Triton X-100 (buffer A), and the column was eluted with the same buffer solution. Fractions containing protein were pooled, and about 300 ml of this solution was incubated for 1 hr at room temperature with 15 μCi of [^{14}C]glucose. The volume of the solution was then reduced by ultrafiltration, using an Aminco Diaflow apparatus, applied to a Sephadex G-200 column equilibrated with buffer A, and the column was eluted with the same buffer solution. The fractions containing the glucose transport-stimulating activity and protein-bound [^{14}C]glucose were desalted by ultrafiltration and applied to DEAE-cellulose columns equilibrated with 5 mM triethanolamine buffer, pH 7.4/2 mM EDTA/2% glycerol/0.02% Triton X-100 (buffer B). After an initial peak was eluted with buffer B, a 0-0.5 M NaCl gradient in buffer B was applied and further fractions containing protein-bound [^{14}C]glucose and the transport-stimulating activity were obtained.

Assay for Glucose Transport. The secondary culture of the chicken embryo fibroblasts was grown in 100-mm plastic dishes

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: RSV, Rous sarcoma virus; NaDodSO₄, sodium dodecyl sulfate.

* Present address: Korea Institute of Science and Technology, Seoul, Korea.

† To whom reprint requests should be addressed.

FIG. 1. Detergent effect on the release from cultured cells of glucose-binding factor and glucose uptake by treated cells. After the culture medium was drained, the confluent secondary culture dishes (100-mm diameter) of chicken embryo fibroblasts were rinsed with two 8-ml portions of Hanks' balanced salt solution, and finally incubated for 30 min at room temperature with 3 ml of 5 mM triethanolamine-HCl/100 mM NaCl containing various amounts of detergents; increasing amounts of octanoate were used in A and Triton X-100 in B. After this incubation, the detergent-containing solution was decanted and used for determining the glucose-binding activity. For this purpose, it was collected from each dish. After the volume was reduced by lyophilization, the sample was applied to a Sephadex G-50 column (12 × 0.7 cm) equilibrated with 5 mM triethanolamine buffer/100 mM NaCl and was eluted with the same solution. Of the 2 ml of protein fraction collected, half was mixed with 0.01 μ Ci of [¹⁴C]glucose (240 Ci/mol) in the same buffer solution. After the sample was kept for 30 min at room temperature, the binding protein was collected on a protein-trapping Millipore filter (HAMK, Millipore Corp.), washed twice with 8-ml portions of the same buffer solution, and dried, and the trapped radioactivity was determined. The remaining half was used for protein determination. For measuring 2-deoxyglucose uptake, the culture dishes were rinsed twice with 8-ml portions of Hanks' salt solution, and [³H]2-deoxyglucose uptake was determined.

in a CO₂ chamber, using the above culture medium, until the cells reached confluency. The dishes were drained and rinsed with three 8-ml portions of Hanks' balanced salt solution. To each dish, either 4 ml of Hanks' balanced salt solution alone or aliquots of the transport-stimulating fraction in 4 ml of the same solution was added. The dishes were preincubated for 30 min at 37°C (1), when 2 μ Ci of [³H]2-deoxyglucose in 0.2 ml of Hanks' solution was added to each dish and incubation was continued for an additional 15 min at the same temperature. The radioactive solution was then decanted. The dishes were rinsed with three 8-ml portions of Hanks' solution, 5 ml of 0.1 M NaOH was added to each dish, and the cells were solubilized. Aliquots of the solubilized cell samples were used for determination of radioactivity and protein content. An assay for [¹⁴C]glucose-binding protein by Millipore filtration is described in the legend of Fig. 1.

RESULTS

A combination of the glucose-binding and transport-stimulating proteins was previously obtained from the resting cells of chicken embryo fibroblast culture dishes by washing the cell surface with an EDTA-containing buffer (1). This washing procedure was found to be only partially effective for testing metabolically more active cells; i.e., cells before and immediately after reaching confluency. To achieve more complete release of the binding-stimulating proteins from the metabol-

ically active cells, various detergents were included in the washing solution. Of those tested, Na octanoate gave satisfactory results because it released the protein without causing cell lysis (Fig. 1A). The other detergents invariably lysed the cells at a concentration sufficient to release the binding protein completely (Fig. 1B). However, for release from RSV-transformed cells, only Triton X-100 could be used. The cell washing fluid obtained with octanoate-containing buffer solution showed a relatively low capacity for binding glucose, which increased severalfold when octanoate and other low molecular weight substances were removed by Sephadex G-50 chromatography.

Partial Chromatographic Separation of Glucose-Binding and Transport-Stimulating Proteins. Chromatography of the [¹⁴C]glucose-labeled cell washing fluid on Sephadex G-200 column containing 0.02% Triton X-100 yielded three protein peaks with both the protein-bound [¹⁴C]glucose activity and the glucose transport-stimulating activity (Fig. 2). However, the amounts of protein-bound [¹⁴C]glucose and transport-stimulating activity did not precisely coincide in all peaks; for example, peak C contained more transport-stimulating than glucose-binding protein activity. More importantly, the bound radioactivity and the transport-stimulating activity only partly overlapped in this peak, which contained a relatively larger quantity of the latter, while the opposite was true for peaks A and B. This suggested the possibility of a chromatographic

FIG. 2. Sephadex G-200 column chromatography of cell wash fluid. About 30 ml of $[^{14}\text{C}]$ glucose-labeled cell wash fluid prepared from 20 roller bottles (57×11 cm) was chromatographed on a Sephadex G-200 column (80×6.5 cm) equilibrated with 5 mM triethanolamine-HCl, pH 7.4/2 mM EDTA/50 mM NaCl/0.02% Triton X-100, using the same solution as eluant. From each fraction, 0.2 ml was used to determine the radioactivity of protein-bound $[^{14}\text{C}]$ glucose and 0.2 ml to determine the 2-deoxyglucose uptake-stimulating activity. The values of uptake represent the uptake with various fractions minus that with no addition.

separation of transport and binding activities. To resolve the transport-stimulating activities and the glucose-binding proteins further, peaks A and C from the Sephadex column were chromatographed on DEAE-cellulose columns containing 0.02% Triton X-100 and 2% glycerol (Fig. 3). As shown in Fig. 3A, upon chromatography on a DEAE-cellulose column, peak A from Sephadex G-200 yielded four peaks with protein-bound $[^{14}\text{C}]$ glucose (α , β , δ , and ϕ) and four peaks with transport-stimulating activity (α , β , γ , and δ). Of these, both the transport-stimulating activity and the radioactivity were most

prominent and overlapping in peak δ , while peak γ contained only the stimulating activity and peak ϕ only the protein-bound glucose. When similarly chromatographed on a DEAE-cellulose column, peak C from Sephadex G-200 was also resolved into peaks with protein-bound glucose (α , δ , and ϕ) and peaks with transport-stimulating activity (α , γ , δ , and ξ). Peak B of Sephadex G-200 (not shown) also yielded peaks α , γ , δ , and ϕ when subjected to DEAE-cellulose chromatography. Therefore, these peaks were present in both the low molecular weight and high molecular weight regions of Sephadex G-200 chromatography, which may indicate that the glucose-binding and transport-stimulating proteins have a tendency to aggregate.

Because serum contains a factor that stimulates glucose transport, a DEAE-cellulose chromatogram of calf serum was prepared in an identical manner to those in Fig. 3A and B; the results are shown in Fig. 3C. Serum yielded the transport-stimulating peaks α , β , a shoulder γ , and ξ , but there is no indication of the presence of a binding protein. It may be concluded, therefore, that the peaks that partially overlap with the transport-stimulating ones in Fig. 3A and B resulted from the chromatography of cell washing fluid originating from adsorbed serum, leaving the appearance of peak ϕ , which contained only binding protein, to be due presumably to a separability of the combined fractions.

From a comparison of the three parts of Fig. 3, it appears that the transport- and binding-stimulating peak δ , which coincides with the largest amount of bound glucose, is absent in serum, indicating that this transport fraction originated from the chicken cells. Peak α contained more than half of the applied proteins, and when rechromatographed on a DEAE-cellulose column, produced mainly peak δ as well as small amounts of peaks γ and ϕ .

Table 1. Conversion of $[^{14}\text{C}]$ 2-deoxyglucose to $[^{14}\text{C}]$ 2-deoxyglucose 6-phosphate

Transport protein, μg	Total $[^{14}\text{C}]$ 2-deoxyglucose taken up, $\text{cpm} \times 10^{-3}$	$[^{14}\text{C}]$ 2-Deoxyglucose-6-phosphate formed, $\text{cpm} \times 10^{-3}$
0	17.2	16.1
2	29.2	27.4
5	41.8	38.6
10	71.1	67.3

The secondary culture of chicken embryo fibroblasts was incubated at 37°C for 15 min with 2 μCi of $[^{14}\text{C}]$ 2-deoxyglucose and various amounts of glucose transport protein. The culture dishes were washed with three 8-ml portions of Hanks' salt solution; the cell samples were transferred to centrifuge tubes and then lysed with 5 ml of boiling 50% aqueous phenol. After the samples were cooled to room temperature, they were centrifuged and the upper aqueous phase was collected. A 0.2-ml aliquot was spotted on Whatman no. 3 paper and the chromatogram was developed with isobutyric acid/water/ammonia (66:33:1 vol/vol) as solvent. Radioactive spots were detected on the chromatogram by means of a radioautogram, and the radioactivity of the spots was determined.

FIG. 3. DEAE-cellulose chromatography of the glucose-binding and transport-stimulating fractions and of calf serum. (A) Peak A from the Sephadex G-200 chromatography of Fig. 2 was desalted and chromatographed on a DEAE-cellulose column (10×1.5 cm). From each fraction, 0.2 ml was used to determine the radioactivity of protein-bound $[^{14}\text{C}]$ glucose and the stimulation of $[^3\text{H}]$ 2-deoxyglucose uptake. The values of uptake represent the uptake with fractions minus that with no addition. (B) Peak C from the Sephadex G-200 chromatography of Fig. 2 was treated as described in A. It was then chromatographed on a DEAE-cellulose column, and fractions were assayed for protein-bound $[^{14}\text{C}]$ glucose and stimulation of $[^3\text{H}]$ 2-deoxyglucose uptake as in A. (C) Calf serum was chromatographed on DEAE-cellulose, and fractions were assayed for stimulation of $[^3\text{H}]$ 2-deoxyglucose uptake as in A.

Interrelation of Deoxyglucose Transport and Phosphorylation. These tests were carried out to show whether stimulation by fibroblast extract of $[^3\text{H}]$ 2-deoxyglucose incorporation into the cell reflects its transport across the cell membrane. Since phosphorylation of deoxyglucose is an intracellular process and has often been shown to coincide with its transport, it was desirable to show that phosphorylation was coincident with transport due to addition of our cell fractions. The results in Table 1 indicate that the increased cellular incorporation of deoxyglucose by addition of the peak δ fraction was indeed directly proportional to the increase in formation of deoxyglucose 6-phosphate.

Effects of Addition of Glucose Transport-Stimulating Fraction on Various Cell Types. Glucose transport is elevated in chicken embryo fibroblasts grown in low-level glucose medium and after RSV-induced transformation (9–12). In Fig. 4, the transport stimulation effect of the peak δ fraction of Fig. 3A on these cells was compared with that on cells grown in a

FIG. 4. Stimulation by glucose-transport protein of 2-deoxyglucose uptake of glucose-fed (O) and fructose-fed (●) normal cells and of RSV-transformed (Δ) cells. The secondary cultures of the glucose-fed normal cells and RSV-transformed cells were grown in a medium composed of 5% calf serum, 0.3% tryptose phosphate broth, and Scherer's nutrient containing 24 mM glucose; the fructose-fed cells were grown in a medium in which tryptose phosphate and glucose were replaced by glucose-free tryptose phosphate and 24 mM fructose. The uptake of $[^3\text{H}]$ 2-deoxyglucose was determined. The starting points on the ordinate indicate uptake before addition.

glucose-rich medium. The rather sizeable background transport in these samples is indicated by the starting points of the three curves on the ordinate, which already show strongly elevated initial levels of glucose transport by fructose-grown and malignant cells as compared to glucose-grown cells. For the latter, the stimulation at saturation was only about 2-fold in these preconfluent cells as compared to 7-fold with earlier used starved cells (1). However, the fructose-grown cells became oversaturated with a very low concentration of added transport protein, approaching the intrinsic transport level of malignant cells but falling to a plateau on addition of higher concentrations. In contrast to normal cells, glucose transport by RSV-transformed cells was very slightly responsive to the added transport protein. This would result if the surface of the transformed cells was completely saturated with the transport protein or if transformation made re-association of the protein with the cell as difficult as its release from the cell surface (1).

Molecular Weight Determination of Glucose-Binding Protein. The molecular weight of $[^{14}\text{C}]$ glucose-binding protein (peak δ of Fig. 3 A or B) was estimated by sodium dodecyl sulfate (NaDodSO₄) gel electrophoresis (Fig. 5). During the purification, it was observed that the ^{14}C label remained tightly bound, and it was used for assay throughout. On electrophoresis with 0.1% NaDodSO₄, two major radioactive bands appeared

FIG. 5. Determination of the molecular weight of glucose-binding protein by NaDodSO₄ gel electrophoresis. The [¹⁴C]glucose-labeled glucose-binding protein fraction from DEAE-cellulose chromatography (peak δ of Fig. 3) was boiled for 2 hr with 0.05 mM Tris buffer/0.3 M glycine/1 mM EDTA/0.5% NaDodSO₄, and the protein sample was subjected to gel electrophoresis using a 10% polyacrylamide gel containing the same buffer. Slices were made from the gel and their radioactivity was determined.

containing a peak of 73,000 molecular weight and a minor one of 55,000 molecular weight. After the protein was boiled for 2 hr with 0.5% NaDodSO₄, these two radioactive bands were converted to 36,000 and 18,000 molecular weight bands. Therefore, it may be concluded that the monomeric form of the protein has a molecular weight of 18,000 and the bands with molecular weights of 36,000, 55,000, and 73,000 should be the dimer, trimer, and tetramer, respectively, of the monomeric form. The nature of the bond between the protein and glucose is not known, but both boiling in NaDodSO₄ and prolonged exposure to the acetic acid contained in staining and destaining solutions results in partial loss of the label.

DISCUSSION

In these experiments, metabolically active, 5% serum-grown cells were used which displayed a higher background of glucose uptake than the quiescent cells used in previous experiments (1). The fractionation of extracts of serum-grown cells yielded glucose-binding and transport-stimulating proteins. The serum contained a fraction which, cochromatographically, partly coincided with separate glucose-transport fractions. It did not contain a glucose-binding protein; however, in the cell chromatograms, a separate peak for glucose binding (peak ϕ of Fig. 3 A and B) appeared, which suggests a chromatographic separability of glucose-binding and -transport proteins. In this respect, experiments on the competition for binding and transport between other sugars and D-glucose or D-deoxyglucose may be indicative. They are being published in detail in a review on this topic (8). These measurements showed an analogous spectrum for both the binding and transport reactions: mannose, glucosamine, and D-glucose competing, and

fructose, galactose, and L-glucose being inactive. For the binding effect, however, the two sugars had to be added together to compete, while the competition for transport was independent of the order of addition. Thus, in binding, but not in transport, with time there appears to be a shift from loose to tight connection with glucose.

These studies were supported by grants from the National Institutes of Health (GM-13972) and from the National Science Foundation (PCM75-21165).

1. Lee, S. G. & Lipmann, F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 163-166.
2. Kawai, S. & Hanafusa, H. (1971) *Virology* **46**, 470-479.
3. Kletzien, R. & Perdue, J. F. (1974) *J. Biol. Chem.* **249**, 3383-3387.
4. Sefton, B. & Rubin, H. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 3154-3157.
5. Bose, B. K. & Zlotnick, B. J. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2374-2378.
6. Shackney, S. E. & Joel, C. D. (1966) *J. Biol. Chem.* **241**, 4004-4010.
7. Weis, L. S. & Narahara, H. T. (1969) *J. Biol. Chem.* **244**, 3084-3091.
8. Lipmann, F. & Lee, S. G. (1978) in *Microenvironments and Cellular Compartmentation*, eds. Srere, P. A. & Estabrook, R. W. (Academic, New York), pp. 263-278.
9. Martineau, R., Kohlbacher, M., Shaw, S. N. & Amos, H. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3407-3411.
10. Kletzien, R. & Perdue, J. F. (1974) *J. Biol. Chem.* **250**, 593-600.
11. Hatanaka, M. & Hanafusa, H. (1970) *Virology* **41**, 647-652.
12. Martin, G. S., Venuta, S., Weber, M. & Rubin, H. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2739-2741.