Phosphorylation of Extracellular Carbohydrates by Intact Cells

CHICKEN HEPATOCYTES SPECIFICALLY ADHERE TO AND PHOSPHORYLATE IMMOBILIZED N-ACETYLGLOUCOSAMINE*

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Cell-cell adhesion is a multi-step process which may be initiated by binding of cell surface carbohydrates to complementary carbohydrate receptors on opposing cell surfaces. We have modeled such interactions using polyacrylamide gels covalently derivatized with glycans, to which intact cells specifically adhere; chicken hepatocytes adhere to gels derivatized with N-acetylglocosamine (GlcNAc). Initially adhesion is blocked (or reversed) by soluble GlcNAc, but becomes sugar-resistant rapidly at 37°C, perhaps due to cellular modification of the carbohydrate-derivatized surface (Guanaccia, S. P., Kuhlnschmidt, M. S., Slife, C. W., and Schnaar, R. L. (1982) J. Biol. Chem. 257, 14293-14299). We report here that, subsequent to recognition and adhesion, intact chicken hepatocytes transfer phosphate covalently to GlcNAc-derivatized gels. Metabolically radiolabeled cells (32P) were incubated on polyacrylamide gels derivatized with various aminohexyl glycosides. Noncovalently bound material was then removed from the gels by extensive washing in detergents and salt solutions. Subsequent radiocchemical analysis revealed that phosphate was transferred selectively to GlcNAc-derivatized gels (up to 20-fold more than to glucose-, galactose-, or mannose-derivatized gels). Soluble GlcNAc (but not other sugars) or low temperature inhibited phosphate transfer. The phosphorylation was mediated by intact cells; cell lysate was itself incapable of specific phosphate transfer and attenuated specific transfer when added to intact cells. When GlcNAc was immobilized using a cleavable (disulfide-containing) linker arm the transferred phosphate radiolabel could be solubilized by disulfide reduction and recovered for further analysis. The released phosphorylated product migrated as a phosphomonoester of GlcNAc. The extracellular carbohydrate phosphorylation reported here may represent one form of intercellular communication.

Specific cell-cell adhesion is a complex multi-step phenomenon in which cell recognition is rapidly followed by cellular responses that strengthen and modify the maturing adhesive bond (1-3). Cell surface carbohydrates and complementary carbohydrate binding proteins on opposing cell surfaces may mediate these cell-cell interactions (1,4). Therefore, we have modeled cell recognition by studying the ability of intact cells to specifically adhere to otherwise inert polyacrylamide gels covalently derivatized with carbohydrates (5,6). In one such system chicken hepatocytes adhered specifically to surfaces derivatized with glycosides of N-acetylglucosamine (GlcNAc) but not other sugars (7,8). Although soluble GlcNAc blocked this adhesion and reversed adhesion after brief incubations, longer incubations at 37°C resulted in the development of sugar resistance (7). Our previous results (9) suggested that sugar-resistant adhesion may be due to covalent modification of the immobilized carbohydrates by intact cells. In the present paper we report that intact chicken hepatocytes specifically phosphorylate surfaces covalently immobilized with GlcNAc. To investigate this reaction further we developed a reversible immobilization reagent (10) which allowed recovery of the covalently immobilized carbohydrates after incubation with intact cells, and identified the phosphorylated species as a phosphomonoester of GlcNAc. The extracellular carbohydrate phosphorylation reported here may represent one form of intercellular communication.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: N-hydroxy succinimide (recrystallized from ethanol) and 4aminobutyric acid, Aldrich; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, diithiothreitol (DTT), N-ethylmaleimide

1 Schnaar, R. L., Langer, B. G., and Brandley, B. K. (1985) Anal. Biochem., in press.
2 The abbreviations used are: DTT, diithiothreitol; NEM, N-ethylmaleimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid; HBS, Hepes-buffered saline; DF-HBS, divalent cation-free HBS; HDMEM, Hepes-buffered Dulbecco's modified Eagle's medium; PF-MEM, phosphate-free minimum essential medium; Temed, N,N,N',N'-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; GalNAc, N-acetylgalactosamine; GlcNAc-6-P, N-acetylglucosamine-6-phosphate; GlcNAc-P, N-acetylgalactosamine phosphate (solution of phosphate undefined). Immobilization reagents (and intermediates) are designated: N-6, N-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxoethyl]-2-propenamide; N-10, N-[4-(2,5-dioxo-1-pyrrolidinyl)-4-oxybutyl]-6-[1-oxy-2-propenyl]amino]-hexanamide; AEMAS, N-[2-[3-[4-[2,5-dioxo-1-pyrrolidinyl]oxy]-4-oxybutyl]6-[1-oxy-2-propenyl]amino]-3-oxopropyl]-dithio[ethyl]-2-propenamide. Glycoside linkers (or controls) are designated: AH-O-Glc, 6-aminoethyl-β-D-gluco pyranoside; AH-O-GlcNAc, 6-aminoethyl-2-acetamido-2-deoxy-β-D-glucopyranoside; AH-S-Gal, 6-aminoethyl-1-thio-1-deoxy-β-D-galactopyranoside; AH-S-Glc, 6-aminoethyl-1-thio-1-deoxy-β-D-galactopyranoside; AH-S-GlcNAc, 6-aminoethyl-2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside; AH-S-Man, 6-aminoethyl-1-thio-1-deoxy-β-D-mannopyranoside.

* This work was supported by National Institutes of Health Grants HD 14010 and CA 21901. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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A longer noncleavable immobilization reagent is designated N-10:

\[
\text{CH}_2\text{CH} - \text{C} - \text{N} - \left(\text{CH}_2\text{CH} - \text{N} - \left(\text{CH}_2\text{CH}_2\text{N} - \text{C} \cdot \text{O} - \text{N}\right)\right)
\]

It was synthesized from N-6 as follows. 4-Aminobutyric acid (0.55 g, 5.3 mmol) was added to 25 ml of water and killed on ice. N-6 (1.5 g, 5.3 mmol) dissolved in 15 ml of ethanol:chloroform (6:1) was added dropwise to the rapidly stirring solution. The pH was monitored continuously and maintained between pH 8 and 9 by addition of NaOH. After the pH became stable, the solution was acidified to pH 2 by addition of HCl and extracted twice with 50 ml of chloroform. The combined chloroform layers were dried over anhydrous sodium sulfate and the solvent evaporated under vacuum. To the resulting residue was added 25 ml of anhydrous ethanol, N-hydroxysuccinimide (0.69 g, 6 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.15 g, 6 mmol). The solution was stirred at 0°C for 2 h. 200 ml of chloroform were added, and the organic layer extracted with 100 ml of saturated aqueous bicarbonate then twice with 200 ml of water. The organic layer was dried over anhydrous sodium sulfate, solvent evaporated under vacuum, and the desired product crystallized from ethyl ether:ethanol. The product (N-10) migrated as one major species on silica gel thin layer chromatography with an Rf of 0.27 using ethyl acetate:acetic acid:water (8:2:1) as solvent (N-6 migrates with an Rf of 0.65 in this solvent). The product reacted positively with 2,4-dinitrophenylhydrazine; treatment of the crude product with 1% HCl afforded a material (compound N-10) that migrated as one species on silica gel thin layer chromatography using ethyl acetate:acetic acid:water (8:2:1) as solvent. An aqueous solution (4 ml) containing 40% (w/v) acrylamide and 2.5% (w/v) bisacrylamide was added and the mixture subjected to rotary evaporation under reduced pressure to remove the ethanol. The resulting aqueous mixture (~3 ml) was incubated with a mixed bed ion exchange resin for 15 min to remove any charged contaminants as described previously (7), eluted, the volume adjusted to 7 ml with water, 10 µl of N,N-methylenebisacrylamide, and the mixture degassed. E. Merck, 6-Aminohexyl glycosides were prepared by published methods (11, 12). All other reagents were of the highest available quality and were obtained through standard sources.

**Carbohydrate-derivative Gel**—Most of the methods for preparation of immobilization reagents, their use in synthesizing carbohydrate-derivative gels, and determination of the immobilized sugar concentration are reported elsewhere (5, 7, 8, 10—13). Additional methods relevant to the experiments described in this paper are detailed below.

Aminohexyl glycoside ligands (11, 12) were immobilized on polyacrylamide gel discs using one of three immobilization reagents, each containing an N-succinimidyl ester at one end, to react with the primary amino group of an aminohexyl glycoside, and acrylyl group at the other end for co-polymerization into acrylamide gels.

Two noncleavable immobilization reagents which differ in the length of the linker arm were used. The synthesis of the shorter reagent, N-6, was reported previously (Compound I, Ref. 16). It consists of the N-succinimidyl ester of 6-acrylamidoxeic acid:

\[
\text{CH}_2\text{CH} - \text{C} - \text{N} - \left(\text{CH}_2\text{CH} - \text{N} - \left(\text{CH}_2\text{CH}_2\text{N} - \text{C} \cdot \text{O} - \text{N}\right)\right)
\]

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NaCl, 1 mM EDTA, 20 mM Hepes buffer, pH 7). Finally, gels were washed in either HBS-8 or distilled water adjusted to pH 8 by addition of ammonium hydroxide, placed in 5 ml of aqueous solution in scintillation vials and gel-associated radiolabel quantitated as Cerenkov radiation in a liquid scintillation spectrophotometer (LKB 1217 Rackbeta). All radiolabel determinations were corrected for efficiency and disintegrations/min.

Release of Phosphate Label—AEMAS-linked ligands (and any associated phosphate radiolabel) were released from the gels by incubation in 2-30 mM DTT in either HBS-8 or distilled water adjusted to pH 8 by addition of ammonium hydroxide (1.7 ml of solution/cm2 evaporation under vacuum at 40 °C and the residue resuspended in a small volume of water). The gels were incubated for 20 min at ambient temperature with intermittent agitation, a procedure which results in the release of 99% of test ligands reversibly immobilized on AEMAS-activated gels. The resulting supernatant solution (containing solubilized ligand and radiolabel) was extracted three times with 3 volumes of ethyl acetate. The DTT was added back to the aqueous phase. All of the radiolabel remained in the aqueous phase during extraction. The volume of the resulting aqueous solution was reduced by rotary evaporation under vacuum at 40 °C and the residue resuspended in a small volume of water. Aliquots of the sample were subjected to chromatographic and electrophoretic analysis as described below. On occasion, N-ethylmaleimide was added (0.7 x) and the sample stored for several days prior to analysis.

Sulphydryl Alkylation—When glycosides were immobilized using the disulfide-containing linker AEMAS and subsequently released by reduction with DTT they contained a free sulphydryl group on the end of the glycoside (see Fig. 9). Spontaneous oxidation to form disulfides (or sulfones) led to changes in chromatographic properties and ambiguity in data interpretation (see "Results"). Therefore, an alternative to the release procedure detailed above was devised which included sulphydryl-specific alkylation with N-ethylmaleimide (19). Washed gels containing the desired phosphorylated product were incubated in 5 mM sodium phosphate buffer, pH 8, containing 5 mM DTT. After 60 min at ambient temperature, 84% of the radiolabel was solubilized. The DTT solution containing the solubilized glycoside was removed from the gels and treated with a 10-fold molar excess (based on the DTT concentration) of NEM for 60 min at 37 °C. Upon extraction with ethyl acetate four times with 4 volumes of NEM, DTT, and alkylated DTT moved quantitatively into the organic phase while the radiolabel remained in the aqueous phase. The volume of the aqueous phase was reduced by rotary evaporation at 40 °C, the residue resuspended in a small volume of water, and aliquots subjected to the chromatographic and electrophoretic analyses described below.

Gel Permeation Chromatography—Solubilized phosphorylated product (see above) was applied to columns of Sephadex G-25 superfine (2 x 20 cm, Pharmacia) or Bio-Gel P-2 (2 x 32 cm, Bio-Rad) and eluted with 150 mM NaCl in 10 mM Hepes buffer, pH 7. Fractions (1.5 ml) were collected in scintillation vials and radioactivity determined after the addition of fluor (Cerenkov radiation).

Paper Electrophoresis—Samples were applied to Whatman 3MM paper and subjected to electrophoresis in pyridineacetic acidwater (5:2:95) at 60 V/cm for 60 min. For preparative paper electrophoresis, the electrophoretogram was dried, the phosphorylated product(s) located with z Geiger counter, cut from the electrophoretogram, and radiolabeled product eluted by descending chromatography in water. Traces of paper electrophoresis coolant (Varsol) were extracted with ethyl acetate and then traces of ethyl acetate were evaporated under a stream of nitrogen. The resulting product was free of undeveloped glycoside and was suitable for thin layer chromatography as described below. For analytical determinations the paper was dried thoroughly, sample bands cut into 0.5-cm strips and placed in scintillation vials. Water (1 ml) and scintillation fluid (6 ml) were added and radiolabel quantitated in a liquid scintillation spectrophotometer (LKB 1217 Rackbeta). As indicated, unlabeled GlcNAc-6-P was added to some analytical samples (1 pmol/lane) before electrophoresis and was located by autoradiography by spraying the electrophoretogram with 0.2 M NaOH in 50% ethanol, heating at 110 °C for 10 min, and examining for fluorescence under ultraviolet light (127).

Cellulose Thin Layer Chromatography—Samples were applied to cellulose TLC plates and developed in one of the following solvent systems: A, isobutyric acidwater (45:55); B, acetonitrileisopropanolwater (3:2:1); C, aqueous 1 M ammonium acetate, 1 mM EDTA/95% ethanol (30:75); and D, 95% ethanol/0.05% aqueous KCl (41). After development, the plates were dried and sample lanes scraped in 0.5-cm segments into scintillation vials. Radiolabel was eluted and quantitated as described above. As indicated, GlcNAc-6-P standard (0.5 pmol/lane) was added to some samples before chromatography and detected as described above.

RESULTS

Carbohydrate-specific Adhesion and Phosphate Transfer

Cell Adhesion to Reversibly Immobilized Glycosides—Our previous data (8, 9) suggested that intact cells adhere to, then covalently modify immobilized carbohydrates which they recognize on an apposing surface. AEMAS was synthesized and characterized primarily to allow recovery of immobilized carbohydrate ligands after exposure to intact cells. Therefore, we determined the ability of intact hepatocytes to adhere, with carbohydrate specificity, to AEMAS-immobilized glycosides. Chicken hepatocytes adhered rapidly and preferentially to AEMAS-surfaces derivatized with AH-S-GlcNAc (Fig. 1), consistent with their known cell surface carbohydrate recognition properties (7). Adhesion was detectable when the density of immobilized GlcNAc was as low as 0.4 μmol/cm2 and was maximal when the density was above 5 μmol/cm2. The adhesion was blocked by addition of soluble GlcNAc (30 mM) to the adhesion medium (but not addition of other soluble sugars), removal of calcium from the adhesion medium, or by pretreatment of the GlcNAc gels with DTT to remove the carbohydrate ligand (data not shown). These data demonstrate that glycosides reversibly immobilized using AEMAS can be recognized by hepatocytes, justifying their use to probe for carbohydrate modification by intact cells.

Selective Phosphate Transfer from Chicken Hepatocytes to GlcNAc-derivatized Gels—When chicken hepatocytes were

![Fig. 1](https://example.com/fig1.png)

**Fig. 1. Specific cell adhesion to reversibly immobilized aminohexyl glycosides.** Polymerization solutions containing 20% acrylamide, 1% bisacrylamide, and various concentrations of AEMAS (see below) were prepared and polymerized between glass plates as described under "Experimental Procedures." The resulting thin gel sheets were cut into small squares (8 x 5 mm) which were washed twice with water for 10 min at 4 °C then incubated in 0.2 M Hepes buffer, pH 8 (50 μl/gel), containing AH-S-GlcNAc at a concentration of 22.5 mM (for gels having the highest sugar density) or 8 mM (for all other gels). After 2 h at 4 °C the gels were thoroughly washed, and used in cell adhesion experiments (16) or analyzed for immobilized sugar concentration. Gels derivatized with the following densities of AH-S-GlcNAc (μmol/cm2) were tested: 0.4 (▲), 0.8 (△), 1.9 (□), 5.4 (□), 8.2 (□), and 27.2 (□) as well as control gels derivatized with 25.4 μmol/cm2 of AH-S-Gal (×). Chicken hepatocytes were prepared as described under "Experimental Procedures," suspended at a concentration of 106 cells/ml, 50 μl of cell suspension added to each gel, and cell adhesion determined (16) at the times indicated.
metabolically radiolabeled with $^{32}$P, then placed on gels reversibly derivatized with GlcNAc or a control ligand (aminohexanol), rapid selective transfer of phosphate label to the GlcNAc-derivatized gels was observed (Fig. 2). While the kinetics of this transfer varied somewhat among cell preparations, it was generally linear for the first 60-90 min and then plateaued. For this reason, most subsequent incubations were performed for 80 or 120 min.

When gels derivatized with various glycosides were used, phosphate transfer was specific for the GlcNAc-derivatized surfaces (Figs. 3 and 4). While selective transfer was observed when glycosides were immobilized using either cleavable

![Fig. 2. Kinetics of phosphate transfer.](image)

**Fig. 2. Kinetics of phosphate transfer.** Radiolabeled chicken hepatocytes were incubated on gels derivatized with AH-S-GlcNAc (7 µmol/cm² gel, filled circles) or control ligand (6-aminohexanol, open circles) under identical conditions. At the indicated times gels were washed extensively as described in the text. Phosphate radiolabel transfer was determined after solubilization from the gels with DTT.

(AEMAS) or noncleavable (N-6 or N-10) linkers, the efficiency of transfer appeared to depend on the length of the spacer arm (Fig. 3A). The greatest amount of phosphate transfer occurred when AEMAS was used, resulting in a maximum polymer to glycoside distance of 26 Å (as determined using molecular models). The use of N-10 resulted in a shorter distance (23 Å) and slightly less phosphate transfer while the use of N-6 resulted in a distance of 17 Å and only a quarter as much phosphate transfer as when AEMAS was used. When aminohexyl glycosides were immobilized by direct acrylylation using acrylyl chloride (7, 8, 15), a maximum polymer to glycoside distance of 10 Å resulted and no selective phosphate transfer was detectable (data not shown). Although phosphate transfer varied markedly with linker length, AH-S-GlcNAc immobilized by any of the above linkers supported cell adhesion.

Phosphate label transferred to GlcNAc immobilized using the cleavable linker (AEMAS) was released by treatment with the reducing agent DTT (Fig. 3B). In contrast, little release occurred under identical conditions from surfaces to which the glycosides were attached by the noncleavable reagents (N-6 or N-10).

While the extent of specific phosphate transfer varied among cell preparations, selective transfer to GlcNAc-derivatized surfaces was always observed when highly viable cell preparations were used (Fig. 4). Transfer to gels derivatized with glucose, galactose, or mannose was only a fraction of that transferred to gels derivatized with the recognized glycoside (GlcNAc), and was generally as low as that transferred to control gels (derivatized with aminohexanol). In most of the experiments reported, aminohexyl S-glycosides were used because of their resistance to cellular glycosidases. Similar results were obtained when aminohexyl O-glycosides were...
used (Fig. 4). Further evidence for the carbohydrate specificity of phosphate transfer was revealed in inhibition studies (Fig. 5). Specific phosphate transfer to GlcNAc-derivatized gels was sharply attenuated when 50 mM soluble GlcNAc was added to the cell suspension prior to incubation on the gels (conditions which block cell-gel adhesion, 7-9). Addition of 50 mM glucose, galactose, or even the closely structurally related GalNAc caused no attenuation of phosphate transfer. Addition of a phosphatase substrate (1 mM AMP) was also without effect. These data demonstrate that phosphate transfer depends on the cells' ability to recognize the immobilized GlcNAc residues. In addition, phosphate transfer was sharply dependent on the concentration of immobilized GlcNAc, as is cell adhesion (13). As shown in Fig. 6, no selective phosphate transfer to GlcNAc-derivatized gels (compared to Glc-derivatized gels) was observed when sugar concentrations of 5 pmol/cm³ gel or less were used. Increasing the sugar concentration to 10 pmol/cm³, however, resulted in maximal phosphate transfer. In contrast, phosphate transfer to Glc-derivatized surfaces did not increase as the sugar density was increased up to the maximum level used (31 pmol/cm³).

Chicken hepatocytes incubated on glycoside-derivatized gels at 0 °C for 90 min showed no selective transfer of phosphate to GlcNAc-derivatized gels (data not shown). Phosphate transfer to both GlcNAc- and Glc-derivatized gels was identical under these conditions and approximately equal to the background phosphate transfer found to Glc-derivatized gels at 37 °C. We have previously reported that chicken hepatocytes bind specifically to GlcNAc-derivatized gels at 0 °C but that subsequent cellular responses such as strengthening and sugar resistance do not occur (8, 9). The current data, therefore, suggest that GlcNAc-specific adhesion is necessary but not sufficient for phosphate transfer.

The above results imply that phosphate label is transferred directly to the immobilized GlcNAc residues. However, an alternate possibility is that a cellular molecule containing both a free sulfhydryl and (radiolabeled) phosphate group undergoes sulfhydryl exchange with the disulfide on the gel surface derived from the AEMAS linker arm. Two experiments argue against this possibility. First, GlcNAc-specific phosphate transfer occurs when glycosides are immobilized using linkers without disulfides (N-10 and N-6, Fig. 3). Second, we directly tested the above alternative by preparing gels having both noncleavable GlcNAc residues (to induce cell adhesion) and free sulfhydryl groups as potential acceptors of the proposed cellular sulfhydryl-containing molecule. They were synthesized by co-polymerizing the acrylated monomer of AH-S-GlcNAc or AH-S-Glc (using N-6, see Fig. 3) and the disulfide-containing monomer AEM (CH=CH-C(=O)-NH-(CH₂)₆-S-S-(CH₂)₆-COOH) into polyacrylamide gels. The gels were subsequently treated with DTT (20 mM, pH 8, 60 min) to generate free sulfhydryl groups from cleavage of the AEM molecules while retaining noncleavable glycosides. While these surfaces supported specific phosphate transfer (11,500 dpm to the GlcNAc gel, 1,300 dpm to the Glc gel), no significant radiolabel (less than 250 dpm) was released from either gel by DTT treatment. These data further support the conclusion that phosphate transfer is directly to the recognized glycoside (see below).

**Intact Cells Are Required for Phosphate Transfer to GlcNAc-derivatized Gels**—Since the immobilized glycosides are relatively close to the polymer backbone (a maximum of 26 Å by molecular modeling), it is unlikely that the glycosides are internalized before phosphorylation. Therefore, phosphate transfer may be mediated by intact cells or by molecules released from the small percentage of lysed cells which are present in our preparations. Three experimental observations support the hypothesis that intact cells are responsible for the observed phosphate transfer.

(i) Selected phosphate transfer increases as cell viability increases. As the experiments reported in this manuscript progressed, we were concerned by the variability of phosphate transfer among different cell preparations using identical gels. After many experiments we compared the characteristics of these various cell preparations with the efficiency of specific phosphate transfer to determine if any correlation existed. The results of the comparison of the initial cell viability (measured as trypan blue exclusion) with the efficiency of phosphate transfer is shown in Fig. 7. Cell preparations with an initial viability of >85% supported GlcNAc-specific phosphate transfer while preparations of <80% viability did not. For this reason, only cell preparations of >85% viability were used in the experiments reported here. Cell lysis during incubation with gels was determined by measuring released lactate dehydrogenase activity in the medium as described previously (7). When cell preparations were >85% viable, their viability remained constant over the entire course of the incubation. The data suggest that highly viable cells are necessary for phosphate transfer to occur and that lysed cells...
are incompatible with selective phosphate transfer (perhaps due to the release of nonspecific phosphatases).

(ii) Cell lysate does not mediate specific phosphate transfer. A more direct test of whether cell lysate mediated phosphate transfer is presented in Fig. 8. Cells were metabolically radiolabeled with $^{32}$P, as described in the text, then split into two portions. One portion was maintained on ice until use while the other was lyzed (>90% of cells broken) by homogenization (40 strokes) in a Dounce homogenizer. GlcNAc-derivatized or control (Glc-derivatized) gels were incubated with either intact or intact cells (Panel A), or a combination of both intact cells and 15% lysate (Panel B). After incubation at 37°C for 90 min the gels were washed thoroughly (see text) and DTT-released radiolabel quantitated. The data are expressed as the ratio between the disintegrations/min transferred to GlcNAc-derivatized gels and the disintegrations/min transferred to Glc-derivatized gels in each experiment. Each data point represents a separate experiment (performed in duplicate to quadruplicate) using a separate cell preparation.

(iii) Cell-conditioned medium cannot support selective phosphate transfer. To test whether phosphate label released from cells into the medium could become associated selectively with glycoside-derivatized gels, the following experiment was performed. Cells were metabolically radiolabeled and incubated under standard conditions on GlcNAc- or Glc-derivatized gels. The medium was carefully removed from above the gels at the end of the incubation, centrifuged briefly (100 x $g$, 3 min) to remove any intact cells, and applied to a second set of gels for an additional 90 min incubation. All gels were washed and treated identically as described under "Experimental Procedures." Marked selective phosphate transfer was observed when intact cells were present while no transfer was observed with the medium from above those cells (data not shown). The above data strongly support the hypothesis that intact cells, not materials released from cells, are responsible for phosphate transfer.

**Characterization of the Phosphorylated Product**

We considered three possibilities to account for the selective phosphate transfer. (i) Cell adhesion to GlcNAc-derivatized surfaces may have resulted in adsorption of released nucleic acids, phosphoproteins, or phospholipids to the gels. (ii) A cellular metabolite containing both a free sulfhydryl and a linker arm. (iii) Specific cell surface enzymes and substrates may have covalently phosphorylated the recognized carbohydrate residues resulting in a phosphomonooester or phosphodiestersugar derivative. The data detailed below demonstrate that the third alternative is most likely, and that the product of extracellular phosphorylation is a phosphomonoester of the immobilized carbohydrate, GlcNAc (Fig. 9).

**Phosphoryl Transfer**

We examined the ability of the phosphorylated product to support select phosphatase transfer. The data are presented in Fig. 7. The dependence of specific phosphate transfer on cell viability. Radiolabeled chicken hepatocyte preparations of the indicated viabilities (determined by trypan blue exclusion) were incubated (60-120 min, 37°C) on gels derivatized with AH-S-GlcNAc or AH-S-Glc at densities $>$10 $\mu$mol/cm$^2$ gel. Gels were then washed thoroughly (see text) and DTT-released radiolabel quantitated. The data are expressed as the ratio between the disintegrations/min transferred to GlcNAc-derivatized gels and the disintegrations/min transferred to Glc-derivatized gels in each experiment. Each data point represents a separate experiment (performed in duplicate to quadruplicate) using a separate cell preparation.
treated with the sulphydryl-specific alkylating agent NEM. This resulted in complete conversion of the faster moving species to a new mobility (0.7 that of orthophosphate) with no effect on the mobility of the slower moving component, suggesting that the faster moving component was the sulphydryl and the slower moving component the disulfide. Treatment of the released phosphorylated product in the presence of DTT (before extraction with ethyl acetate) with a 10-fold excess of NEM (based on the DTT present) resulted in a single component (upon paper electrophoresis, Fig. 10, upper right panel) which was stable upon storage at 4 °C. Alkylation eliminated chromatographic instability and ambiguity and allowed the more detailed structural studies described below.

Fig. 9. Proposed events in extracellular carbohydrate phosphorylation and its analysis. Aminohexyl glycosides of N-acetylglucosamine were immobilized on polyacrylamide surfaces (A) using the disulfide-containing linker arm, AEMAS (10). Chicken hepatocytes were metabolically radiolabeled with 32Pi and incubated on the derivatized surfaces resulting in extracellular phosphate transfer to the immobilized carbohydrate (B). The presence of the cleavable disulfide bond (arrow, B) allowed subsequent solubilization of the phosphorylated product by mild reduction with DTT. The soluble product (C) was a phosphorylated glycoside of N-acetylglucosamine containing a free sulphydryl at the terminus of its aglycone. The glycoside was either directly analyzed by electrophoresis and chromatography, or the free sulphydryl was alkylated prior to analysis. The O-glycosidic linkage (arrow, C) was susceptible to acid hydrolysis under conditions which did not cleave the phosphomonoester, resulting in conversion of the phosphorylated species to GlcNAc-P (D). Although the figure depicts the site of the phosphate ester as the 3 or 4 position, the data are also consistent with esterification at the 3 or 4 position.

Fig. 10. Kinetics of acid hydrolysis of the phosphorylated product. Phosphorylated product was alkylated and purified by preparative paper electrophoresis as described under “Experimental Procedures.” An aqueous aliquot containing ∼15,000 dpm was treated with 1 N HCl at 100 °C. At the times indicated, portions (∼2,000 dpm) were removed and applied to cellulose TLC plates for development in Solvent A (left panels) or to Whatman 3MM paper for paper electrophoresis (right panels). Developed chromatograms and electrophoretograms were analyzed as described under “Experimental Procedures.” Data are expressed as the disintegrations/min recovered in each fraction as a per cent of the total recovered (80–95% of the disintegrations/min applied). The solid bar indicates the position of standard GlcNAc-6-P and the open bar that of 32P.

Acid Hydrolysis of the Phosphorylated Product—Phosphate radiolabel was specifically transferred from intact hepatocytes to either the O-glycoside or S-glycoside of GlcNAc, and the phosphorylated product solubilized with DTT as outlined in Fig. 9. Subsequent acid hydrolysis of the purified O-glycoside was performed under conditions which leave hexose 6-phosphates intact (22). The kinetics of acid hydrolysis of the O-glycoside are shown in Fig. 10. As determined by cellulose TLC (solvent A) and paper electrophoresis, acid hydrolysis of the phosphorylated product resulted in its disappearance and the concomitant appearance of a new product which co-migrated with GlcNAc-6-P. Further hydrolysis resulted in appearance of label which co-migrated with inorganic orthophosphate. Little or no hydrolysis product co-migrated with glucosamine 6-phosphate which migrated near the origin in both systems. To model the hydrolysis, aminohexyl O-GlcNAc was treated with 1 N HCl at 100 °C and the products subjected to TLC in solvents A, B, and C (data not shown). Within 15 min all of the glycoside was cleaved, releasing almost exclusively free GlcNAc. Further hydrolysis resulted in deacetylation of the GlcNAc to glucosamine. The above results are consistent with the identification of the solubilized phosphorylated product as a phosphomonoester of the glycoside of GlcNAc (Fig. 9C). However, further characterization of the hydrolysis product was performed to establish this conclusion.

Based on the kinetics of acid hydrolysis, an incubation time was chosen (30 min) which resulted in maximum conversion to the proposed phosphomonoester. The hydrolysate was applied to cellulose TLC plates and chromatographed (next to unhydrolyzed sample) in four different TLC solvent systems (Fig. 11). In all four systems (as in paper electrophoresis) most of the unhydrolyzed sample migrated as a single component, while most of the hydrolysate product co-migrated with authentic GlcNAc-6-P.
addition, there was no intermediate breakdown product co-migrating with GlcNAc-6-P when the S-glycoside was used. These data suggest that the cells transferred phosphate directly to the immobilized carbohydrate.

Alkaline Phosphatase Hydrolysis of the Phosphorylated Product—If, as the above data suggest, the phosphorylated product is a phosphomonoester of a GlcNAc glycoside, it should be susceptible to hydrolysis by alkaline phosphatase. When released product was treated with alkaline phosphatase (5 μg/ml in 10 mM glycine buffer, pH 9.8, with 1 mM MgCl₂, 37 °C, 60 min) it was completely converted to a species co-migrating with inorganic orthophosphate (data not shown). These data confirm that the phosphorylated product is a phosphomonoester.

**DISCUSSION**

Cell-cell adhesion is a complex process initiated by specific cell-cell recognition and followed by multiple responses including strengthening of the adhesive bond and its maturation by elaboration of new molecules at the site of cell-cell contact (1–3, 23). Cell surface carbohydrates and complementary carbohydrate binding proteins (lectins) on opposing cells have been implicated as cell-cell recognition molecules in several systems (24–32). Therefore, we have modeled such interactions by studying the adhesion of intact cells to otherwise inert plastic surfaces covalently derivatized with carbohydrate ligands (5–9, 33–38). In one such system, chicken hepatocytes adhere specifically to polyacrylamide gels derivatized with glycodies of N-acetylgalactosammines (7). Carbohydrate-specific cell recognition of, and adhesion to these gels occurs readily at temperatures between 0 and 37 °C, and is rapidly followed (at 37 °C but not at 0 °C) by at least two post-recognition responses (8, 9). The first response is strengthening (>15-fold) which is blocked by metabolic inhibitors. The second response we refer to as sugar resistant. Initial GlcNAc-specific adhesion can be blocked or reversed by the addition of soluble GlcNAc (>1 mM) to the adhesion medium. After incubation for 30–60 min at 37 °C the cells become resistant to release by soluble sugar (7). Our previous data (9) suggested that cells may covalently modify the carbohydrate-derivatized surface, leading to an altered type of adhesion.

A direct test for extracellular covalent carbohydrate modification required recovery of immobilized carbohydrate ligands after interaction with intact cells under conditions gentle enough to retain potential covalent modifications such as sulfation, phosphorylation, glycosylation, etc. Our previous technology had been directed toward stable covalent immobilization and precluded recovery of carbohydrates from the derivatized surfaces except by harsh chemical treatments. Therefore, we developed new technology to immobilize aminecontaining glycosides via linkers containing cleavable disulfide bonds (10). Using the reversible immobilization reagent AEMAS, we obtained direct evidence for extracellular covalent modification of specific immobilized carbohydrates by intact cells. Chicken hepatocytes radiolabeled with 32P were incubated in surfaces covalently derivatized with various carbohydrate ligands transferred radioactivity preferentially to surfaces derivatized with the recognized carbohydrate, GlcNAc. The transfer was sugar specific, required a "threshold" concentration of immobilized GlcNAc (as does cell adhesion), required intact cells, and was not mediated by cell lysate.

The data indicate that the cells directly modify the immobilized glycoside, resulting in the formation of a phosphomonoester of GlcNAc. Although the phosphorylated product was recovered in such small mass quantities that only radiochem-
ical detection was possible, the co-migration of its acid hy-
drolysis product with GlcNAc-6-P in five different separation
systems (Figs. 10 and 11) strongly suggests its identity as a
phosphomonoester of GlcNAc. Since it is likely that the 3- or
4-phosphate ester would behave identically to the 6-phosphate ester upon electrophoresis or chromatography, we cannot
assign the position of the phosphate ester on the carbohydrate
ring as yet. However, the products and kinetics of acid hy-
drolysis favor tentative assignment to the 3- or 4-position.
Model hydrolysis of authentic GlcNAc-6-P resulted in com-
plete deacetylation of the carbohydrate (to glucosamine 6-
phosphate) without apparent dephosphorylation after 2 h in 1
N HCl at 100 °C. In contrast, the phosphorylated product
generated in our studies was largely dephosphorylated under
the same conditions and little or no glucosamine 6-phosphate
breakdown product was detected.

Phosphate esters of carbohydrates have been implicated as
intracellular sorting signals on glycoproteins (37, 38). The
biosynthesis of mannose phosphate residues on certain hy-
drolytic enzymes is thought to be responsible for their target-
ing to the lysosome, and presents one possible mechanism for
phosphorylation of glycosides on pre-formed oligosaccharides
(38). Transfer of a sugar phosphate from a high energy phos-
phate intermediate (UDP-GlcNAc) to the carbohydrate resi-
due (mannose) in the oligosaccharide leads to the formation
of a sugar-oligosaccharide phosphodiester. In a second step,
the terminal sugar (GlcNAc) is removed, leaving a phospho-
monoester of the oligosaccharide. Although only phospho-
monoester was detected in our system, the above mechanism
is not ruled out since a short-lived phosphodiester may exist
at sub-detectable levels.

Unlike the system described above, our data suggest that
carbohydrate phosphorylation can occur at the external sur-
face of intact cells. This requires the postulation of a high
energy phosphate intermediate (as well as the appropriate
phosphotransferase) at the cell surface. A precedent for the
transfer of high energy phosphate intermediates from the
cytosol across a membrane is found in the dolichol phos-
phate intermediates involved in glycoprotein synthesis. Has-
elbeck and Tanner (39) have presented evidence that the
enzyme which synthesizes dolichol phosphate mannosyl
has the potential to flip from a cytoplasmic to a cell surface
orientation. Other mechanisms may involve phosphate trans-
fer from soluble secreted phosphate intermediates, or transfer
via an enzyme-bound high energy phosphate. The experi-
ments described here do not address this point. In any case,
the presence of a cell surface enzyme (or enzymes) responsible
for carbohydrate phosphorylation are strongly implicated
by the data in this paper.

Several laboratories have reported the presence of cell
surface enzymes, although few have unambiguous evidence
for their cell surface localization (41-45). One of the most
broadly studied classes of putative cell surface enzymes are
the glycosyltransferases (41). Proof of their cell surface local-
ization has been elusive since, unlike the phosphate transfer
reported here, most of the glycosyltransferase activity is lo-
cated intracellularly (46) and can be readily released by cell
lysis (47). Recent studies (48, 49), however, have demon-
strated the presence of a protein immunologically cross-re-
active with galactosyltransferase at the cell surface of several
cell types. Although protein kinases have also been reported
to exist on the exterior of cells, the role of cell lysis in
generating that activity has not been ruled out (50, 51).

Cell surface hexokinase activity has not been previously
reported, and its function can only be speculated. However,
phosphorylated mannose residues on glycoproteins (see above)
are responsible for the intracellular targeting of certain
lysosomal hydrolases (37), and phosphorylated glucose resi-
dues have been implicated in binding of cell surface proteins
to ligatin (52). Thus, there is precedent for phosphorylated
glycoconjugates at the cell surface to be involved in recogni-
tion phenomena.

Using the same cells under study here (chicken hepatocytes)
Roseman and co-workers (53) have identified a specific cell-
cell adhesion factor which appears to contain a phosphoryl-
ated GlcNAc residue. In our system, adhesion to the GlcNAc-
derivatized gels appears to be a necessary but not sufficient
prerequisite for phosphate transfer. Soluble GlcNAc blocked
both cell adhesion and phosphate transfer, and both phenom-
ena required a threshold concentration of immobilized
GlcNAc on gels (although the threshold concentration for
phosphate transfer was 10-fold higher than that for cell adhe-
sion). At 0 °C, where cells adhere only weakly (8), no phos-
phate transfer was detected. However, when GlcNAc was
immobilized on gels using a short linker arm (<10 Å) cells
developed sugar-resistant cell adhesion (9) but no measurable
phosphate transfer occurred. Using longer linker arms (up to
>26 Å) phosphate transfer increased with increasing linker
length (Fig. 3). GlcNAc immobilized by the longer spacer
arms was also more efficient in supporting cell adhesion, as
evidenced by a lower threshold for cell adhesion (compare with
Fig. 1, Ref. 8). Since GlcNAc immobilized via the shorter
linker arm supports sugar-resistant adhesion but not phos-
phate transfer, two interpretations are possible; (i) phosphate
transfer may be unrelated to sugar-resistant adhesion; or (ii)
sugar-resistant adhesion may be supported by phosphorylated
sugars at densities below our detection level for radiolabel
phosphate transfer, necessitating the more efficient (longer
linker) sugars for detection of transfer. Resolving these alter-
avatives will require further identification of the phosphoryl-
ated structure, its synthesis, and direct testing of its ability
to support sugar-resistant hepatocyte adhesion.

The role of extracellular carbohydrate phosphorylation re-
 mains obscure. Our previous work (9) suggested that initial
adhesion to GlcNAc-derivatized surfaces occurs via the
chicken hepatic glycoprotein receptor described by Ashwell
and co-workers (54, 55) which is proposed to be responsible
for receptor-mediated endocytosis. No enzyme activity has,
as yet, been attributed to this receptor, although it is phos-
phorylated on itself on the cytoplasmic side of the membrane
(56). The possibility exists that the phosphorylation event we
have documented is involved in receptor-mediated endocyto-
sis and is merely “trapped” outside the cell by immobilization
of the carbohydrate ligand. However, incubation of cells on
GlcNAc-derivatized surfaces under conditions which optimize
phosphate transfer also leads to an alteration in cell adhesion
to these surfaces (8, 9). It is possible that extracellular car-
bohydrate phosphorylation is involved in cell-cell communi-
cation or in the maturing of intercellular adhesions. Only
additional experiments can determine whether extracellular
carbohydrate phosphorylation functions in cell-cell adhesion,
endocytosis, or other processes. Nevertheless, the covalent
modification of carbohydrate residues on an apopposing surface
reported here may represent a mechanism by which cells
communicate with their neighbors or alter the extracellular
matrix.
Acknowledgments—We are grateful to Salahudeen Abdulla Muhammad for aid in cell preparation, Alan Lattimore for experimental assistance, and Paula Manzuk for manuscript preparation.

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