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HEPATOCYTES MEDIATE COENZYME A TRANSFER TO SPECIFIC CARBOHYDRATE-DERIVATIZED SURFACES

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SUMMARY: Freshly isolated chicken and rat hepatocytes adhere with carbohydrate specificity to surfaces derivatized with N-acetylglucosamine and galactose respectively. Previously (Brandley, B.K. and Schnaar, R.L. (1985) *J. Biol. Chem.* **260**, 12474-12483) we reported that metabolically radiolabeled chicken hepatocytes covalently transferred phosphate radiolabel specifically to N-acetylglucosamine-derivatized surfaces. We now report that rat hepatocytes transfer phosphate radiolabel specifically to galactose-derivatized surfaces. Transferred radiolabel from both species to their appropriate carbohydrate-derivatized surface was identified as CoASH. After specific adhesion via the appropriate carbohydrate, CoASH is apparently released from cells and undergoes disulfide exchange with the cleavable immobilization linker we used to tether the sugars to the artificial surfaces. Although CoASH from lysed cells can undergo similar disulfide exchange, the data suggest that other, perhaps physiological mechanisms may be responsible for the carbohydrate-specific radiolabel transfer.

INTRODUCTION: Chicken and rat hepatocytes adhere to polyacrylamide gels covalently derivatized with glycosides of GlcNAc and Gal respectively, but not other sugars (1-3). Appropriate soluble sugars block this adhesion, and reverse adhesion after brief incubations. However, after longer incubations at 37°C cells are no longer susceptible to release by soluble saccharides (4). One possible explanation for sugar resistance, covalent modification of the derivatized surface by the intact hepatocytes, was tested using an immobilization reagent containing an internal disulfide so that the immobilized saccharides could be recovered after incubation with radiolabeled intact cells. Metabolically radiolabeled ($^{32}P_i$) chicken hepatocytes transferred phosphate radiolabel specifically to surfaces derivatized with GlcNAc (5). We report extension of these studies to rat hepatocytes, which transfer phosphate radiolabel specifically to Gal-derivatized surfaces. Initial studies indicated that phosphate radiolabel was transferred directly to the recognized sugar moiety. We now report chemical identification of the transferred species as CoASH.

Abbreviations used: AEMAS, N-[2-[[3-[[4-[(2,5-dioxo-1-pyrrolidinyl)oxy]-4-oxobutyl]amino]-3-oxopropyl]dithio]ethyl]-2-propenamide; DTT, dithiothreitol; NEM, N-ethylmaleimide.

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METHODS

Aminohexyl glycosides were synthesized (6) and covalently, but reversibly immobilized on 0.4 mm thick, 1.4-cm diameter gel disks using AEMAS, a disulfide-containing acrylic immobilization reagent (5,7). Gels containing the indicated concentrations of bound glycoside were thoroughly washed and pre-equilibrated with medium (see below) before incubation with cells. Hepatocytes were prepared by collagenase perfusion of juvenile chicken and rat livers (8), and the resulting cell populations were enriched for viable cells by density centrifugation (9). Cells were suspended at 2 x 10^6 cells/ml for rat hepatocytes and 5 x 10^6 cells/ml for the smaller chicken hepatocytes. Cell suspensions in 15-20 ml of phosphate-free minimal essential medium were supplemented with 0.5-2 mCi of carrier-free $^{32}P_i$, and incubated in rotation culture (125 rpm) for 90 min at 37°C (5). Radiolabeled cells (0.5 ml) were placed on derivatized gels in 24-well dishes and incubated without agitation for 90-120 min at 37°C. At the end of the incubation, the gels were rinsed three times with medium, placed in a detergent solution (1% SDS and 1% Triton X-100 in 20 mM Hepes pH 7.0) and sonicated at ambient temperature for ≈10 min with five changes of detergent solution. After the fifth wash, the gels were boiled in fresh detergent solution for 10 min, rinsed extensively in saline (0.9% NaCl, 20 mM Hepes pH 7.0), and incubated overnight in saline at 4°C. Gel-associated ^{32}P was quantitated using a liquid scintillation spectrometer in the absence of fluor (via Cerenkov radiation).

Most (80-90%) of the radiolabel associated with the gels after the wash protocol was subsequently released upon incubation in dithiothreitol (20 mM DTT in water adjusted to pH 8 with ammonium hydroxide) at ambient temperature for 60 min. The resulting sulfhydryls were alkylated with a 10-fold excess of N-ethylmaleimide (NEM), and the excess NEM, DTT, and alkylated DTT removed by extraction with ethyl acetate (5). Solubilized radiolabel was purified by anion exchange HPLC (Altex Spherogel-TSK DEAE-5PW, 7.5 x 75 mm) in ammonium acetate buffer as indicated in the figure legends, lyophilized, and stored at -20°C. Radiolabeled species were detected after analytical HPLC by collecting fractions and determining radioactivity by liquid scintillation spectroscopy. Unlabeled standards were detected after HPLC using an in-line ultraviolet spectrophotometer.

NEM-alkylated CoASH standard was synthesized by treating CoASH with DTT prior to alkylation using excess NEM or [^{3}H]NEM. Excess reagents were removed by extraction with ethyl acetate, and the concentration of product determined by absorbance at 260 nm (CoASH as standard).

RESULTS AND DISCUSSION: Intact metabolically radiolabeled rat hepatocytes, which possess a Gal/GalNAc binding lectin and which bind specifically to Gal-derivatized surfaces, transferred radiophosphate specifically to surfaces derivatized with galactose, when glycosides were immobilized via AEMAS, a disulfide-containing reversible covalent linker (Figure 1). Except for the sugar specificity, radiolabel transfer was similar to that by chicken hepatocytes to immobilized GlcNAc (5). The immobilized galactose density required to support phosphate transfer from rat hepatocytes to Gal gels (55 μmol/ml) was considerably higher than that required to support rat hepatocyte adhesion to these surfaces

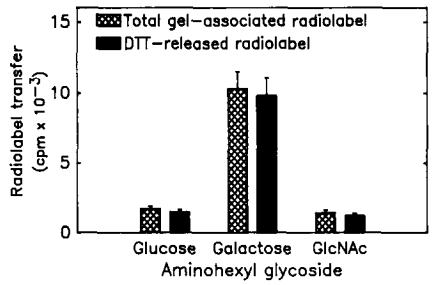

Figure 1. Carbohydrate-specific phosphate transfer from intact rat hepatocytes to Gal-derivatized surfaces. Highly viable rat hepatocytes were radiolabeled with $^{32}P_i$ and placed on polyacrylamide gel discs derivatized with 50-60 μmol/cm^3 of the indicated aminohexyl glycosides. After 120 min at 37°C cells were removed by rinsing vigorously with medium, and the gels washed and boiled with detergents as described in Methods. Radiolabel associated with the gels was determined without the addition of fluor (Cerenkov radiation) prior to recovery of the radiophosphorylated species by DTT treatment. Data from triplicate determinations in four separate experiments are presented as the mean ± S.E.M.

($\approx$10 μmol/ml). This can be compared to the chicken hepatocyte/GlcNAc system, where 10 μmol/ml of immobilized GlcNAc is required to support radiophosphate transfer, while <2 μmol/ml GlcNAc supports cell adhesion. Chemical analysis of recovered radiophosphate revealed the same molecular structure transferred in both systems.

Specifically and covalently transferred radiophosphate was recovered by DTT treatment of carbohydrate-derivatized surfaces which had been incubated with ^{32}P-labeled hepatocytes (chicken hepatocytes on GlcNAc gels or rat hepatocytes on Gal gels). The radiolabel released from the two systems was chemically indistinguishable. Mild acid treatment (0.1 N HCl, 15 min, 90°C) revealed a previously undetected hydrolysis product which suggested that the phosphorylated species was more complex than the phosphomonoester initially proposed. The chromatographic profile of the radiolabeled species was sensitive to both phosphodiesterase (see below) and alkaline phosphatase, but not to any other enzymes tested, including exoglycosidases. Anion-exchange HPLC revealed the product to be highly anionically charged. Comparison of the HPLC mobility of the phosphorylated species with that of standard compounds on both DEAE (Altex) and strong anion exchange (Alltech Adsorbosphere SAX 5μ, 250 x 4.6 mm) columns revealed co-migration with NEM-alkylated CoASH (Fig. 2). All other chromatographic systems tested, including reverse phase HPLC (Vydac Reverse Phase C_{18}), Bondapak NH_2 HPLC (Waters), Dowex AG-1X8, gel permeation chromatography on Bio-Gel P-2 (Bio-Rad), and thin layer chromatography on cellulose and silica gel layers also resulted in co-migration of the radiophosphate with NEM-CoASH (data not shown). Furthermore,

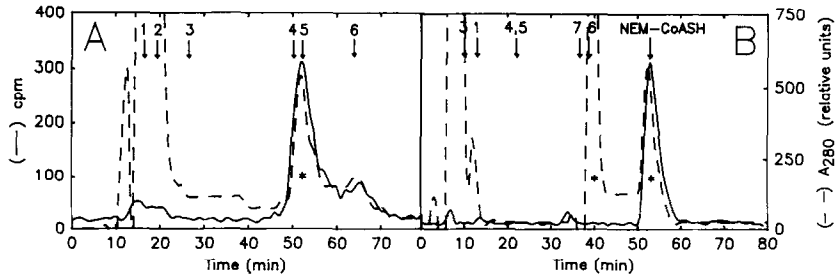

Figure 2. Co-migration of transferred radiophosphate recovered from Gal surfaces with NEM-alkylated CoASH. The radiophosphorylated species covalently transferred from $^{32}P_i$-labeled rat hepatocytes specifically to Gal-derivatized gels was recovered by DTT-treatment, NEM-alkylated and purified by HPLC as described in Methods. Purified radiolabel was mixed with authentic NEM-alkylated CoASH (*panel A*) or with a mixture of CoASH and NEM-alkylated CoASH (*panel B*) and chromatographed by HPLC on a DEAE (Altex) column (*panel A*) or a strong anion exchange (SAX) column (*panel B*). Solid lines represent the radiolabel elution profile, while dashed lines represent the U.V. absorbance at 260 nm. Peaks with an absorbance ratio (260/280 nm) characteristic of nucleotides are indicated with asterisks. The mobilities of other standards, indicated by numbered arrows, were determined from independent HPLC runs and are shown according to their mobility relative to NEM-alkylated CoASH: (1) NADP; (2) Pi; (3) IP_2; (4) ATP; (5) IP_3; (6) CoASH; (7) IP_4.

the alkylated phosphorylated product and NEM-alkylated CoASH were equally sensitive to acid hydrolysis, to acetylating agents, and to phosphodiesterase treatment (Fig. 3, left panel), generating products with the same altered mobility. The unalkylated radiophosphate released from gels co-migrated with CoASH and was equally susceptible to enzymatic acetylation by pyruvate dehydrogenase (Fig. 3, right panel). Finally, mild acid hydrolysis profiles of radioligands released from AH-O- and AH-S-glycoside-derivatized gels were compared and found to be identical, suggesting that the phosphorylated product released from the gels no longer contained the original glycosidic linkage.

Chicken hepatocyte preparations that were less than 85% viable were incapable of phosphate transfer to GlcNAc-derivatized surfaces, and neither cell-conditioned medium nor 15% cell lysate supported radiophosphate transfer in that system (5). In contrast, cell lysate from rat hepatocytes did support phosphate transfer, although not with the carbohydrate specificity detected in intact cells. A highly viable preparation of metabolically radiolabeled rat hepatocytes was split into two portions. One portion was homogenized in a Dounce homogenizer fitted with a tight glass pestle until intact cells were nearly absent by microscopic examination. The ability of viable cells, viable cells supplemented with 15% added lysate, 15% added lysate alone, and 100% lysate to support CoASH transfer to carbohydrate-derivatized surfaces was compared (Fig. 4). Only gels

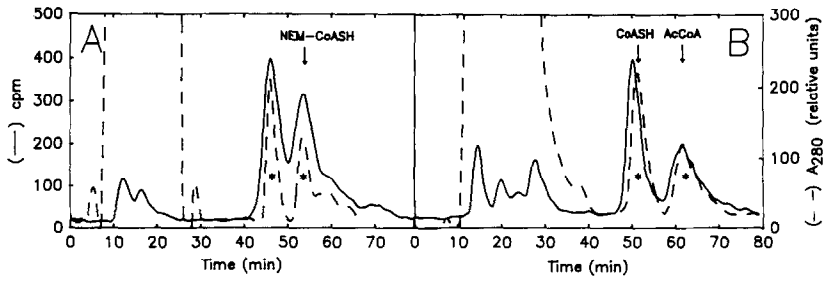

Figure 3. Enzyme treatments of phosphorylated ligand. *Panel A*: Radiophosphate recovered after incubation of Gal-derivatized surfaces with $^{32}P_i$-labeled rat hepatocytes was NEM-alkylated, mixed with standard NEM-alkylated CoASH, treated with 1 mU/ml phosphodiesterase I for 15 min at 37°C (conditions determined to result in incomplete hydrolysis) and analyzed by DEAE anion exchange HPLC. *Panel B*: Radiophosphate recovered (using DTT) after incubation of GlcNAc-derivatized surfaces with $^{32}P_i$-labeled chicken hepatocytes (unalkylated) was mixed with authentic CoASH, incubated with 5 mU/ml pyruvate dehydrogenase at ambient temperature for 60 min, and analyzed by DEAE anion exchange HPLC. In both panels, the solid lines represent elution of radiolabel and the dashed lines represent the U.V. absorbance profile (260 nm). Asterisks denote peaks with absorbance ratios (260/280 nm) characteristic of nucleotides. The arrow in panel A denotes the mobility of NEM-alkylated CoASH and the arrows in panel B indicate the mobilities of CoASH and acetyl CoA determined on separate HPLC runs.

incubated with viable cells (and up to 15% added cell lysate) supported sugar specific phosphate transfer to galactose-derivatized surfaces. However, rat hepatocyte lysate transferred considerable phosphate radiolabel equally to galactose- and control (glucose-) derivatized surfaces. Most of the transferred radiolabel was recovered by DTT treatment, alkylated, and was found to co-migrate with NEM-alkylated CoASH upon anion exchange HPLC. The markedly higher CoASH transfer from lysed rat hepatocytes compared to chicken hepatocytes remains unexplained. One possibility is that, compared to rat

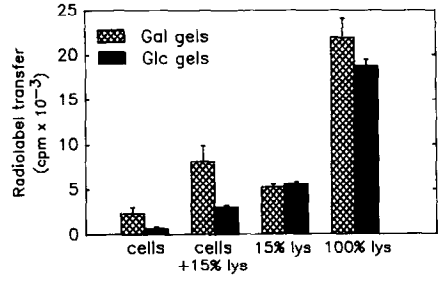

Figure 4. Effect of rat hepatocyte viability on CoASH transfer to Gal-derivatized gels. Highly viable (94%) $^{32}P_i$-labeled rat hepatocytes were split into two portions. One portion was homogenized as described in the text. The ability of the viable cell suspension, viable cells supplemented with 15% cell lysate, 15% cell lysate alone, or 100% cell lysate alone to support radiophosphate transfer to Gal-derivatized gels were compared. Radiolabel transfer per gel (triplicate determinations, mean ± S.D.) are shown.

hepatocyte lysate, chicken hepatocyte lysate has higher phosphatase and/or phosphodiesterase activities which hydrolyze any transferred CoASH prior to analysis. However, measurement of hydrolytic activities against *p*-nitrophenyl phosphate and bis(*p*-nitrophenyl) phosphate in such lysates revealed comparable activities for comparable cell volumes (data not shown). The presence of a highly active, more specific phosphodiesterase in chicken hepatocyte lysates has not been ruled out.

Two key observations helped clarify our previous results (5), which had indicated modification of immobilized GlcNAc by chicken hepatocytes. First, during more recent efforts to rid the gels of all noncovalently-linked material it was discovered that the earlier procedure of sonicating and boiling in 1% SDS solution (without Triton X-100), surprisingly left radiolabeled phospholipids adsorbed to gel surfaces derivatized using hydrophobic linker arms. This may have accounted for the earlier observation that sugar-specific radiophosphate transfer by chicken hepatocytes to GlcNAc-derivatized surfaces was *not* dependent on the use of a disulfide-containing immobilization reagent (5). Second, earlier purification of the transferred radiolabel utilized preparative paper electrophoresis in pyridine acetate buffer (5), which altered the chromatographic behavior and hydrolysis susceptibility of the ligand as well as standard NEM-alkylated CoASH (data not shown).

Taken together, these data suggest that radiolabel transfer from $^{32}P_i$-labeled hepatocytes to carbohydrate-derivatized surfaces is an artifactual consequence of disulfide exchange between CoASH released from lysed cells and the disulfide engineered into the reversible immobilization reagent. However, certain key observations are not so readily explained. If CoASH transfer is due to a small amount (<10%) of non-specific cell lysis: (i) Why is transfer from intact cells sugar specific? (ii) Why don't phosphatases and phosphodiesterases in the small amount of chicken hepatocyte lysate eliminate CoASH transferred from "intact" cells? (iii) Why is there such a sharp dependence of CoASH transfer on sugar density (5), and why is the threshold concentration for CoASH transfer higher than that for cell adhesion? and (iv) Why is the threshold density for rat hepatocyte CoASH transfer so much higher than that for chicken hepatocytes? Although the observation may still be artifactual, these remaining enigmas suggest the intriguing possibility that CoASH export may reflect a physiologically relevant event.

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