Affinity Labeling of the Carbohydrate Binding Site of the Lectin Discoidin I Using a Photoactivatable Radioiodinated Monosaccharide†

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ABSTRACT: *N*-(4-Azidosalicyl)galactosamine (GalNASA), a photoactivatable, radioiodinatable analogue of *N*-acetylgalactosamine (GalNAc), has been prepared and characterized. We have used this reagent for labeling of the carbohydrate binding site of discoidin I, an endogenous lectin produced by *Dictyostelium discoideum*. GalNASA behaved as a ligand for discoidin I, as judged by its ability to compete in an assay measuring the carbohydrate binding activity of discoidin I. In this assay, it exhibited a $K_{i,app}$ of 800 μM, comparable to that of GalNAc. The $K_{i,app}$ of GalNASA decreased to 40 μM upon prior photolysis with ultraviolet light. In contrast, *N*-(4-azidosalicyl)ethanolamine produced no inhibition of carbohydrate binding regardless of photolysis. Covalent labeling of discoidin I with ^{125}I-GalNASA was entirely dependent upon ultraviolet light. A portion of the labeling, representing 40–60% of the total, was sensitive to reagents which were known to inhibit carbohydrate binding by discoidin I, including GalNAc, asialofetuin, and ethylenediaminetetraacetic acid. *N*-Acetylglucosamine, which is not a ligand of discoidin I, was without effect. As a control, no carbohydrate-sensitive labeling was observed upon incubation of ^{125}I-GalNASA with bovine serum albumin. The carbohydrate-sensitive fraction of discoidin I photolabeling with ^{125}I-GalNASA exhibited a K_d of 15–40 μM, in agreement with the $K_{i,app}$ of prephotolyzed GalNASA observed in the carbohydrate binding assay. Some labeling occurred if ^{125}I-GalNASA was photolyzed prior to incubation with discoidin I, suggesting the involvement of long-lived species in the labeling reaction. Partial proteolytic digestion of photolabeled discoidin I revealed specific fragments whose labeling was completely blocked by GalNAc. This indicated that the location of carbohydrate-sensitive labeling within the structure of discoidin I was restricted. One particular tryptic fragment, Tr1, was examined in detail. Photolabeling of this fragment displayed a specificity and sensitivity to carbohydrate competitors identical with that observed for discoidin I in the carbohydrate binding assay. These data suggest that Tr1 is derived from the carbohydrate binding site of discoidin I.

During the aggregation stage of starvation-induced development, *Dictyostelium discoideum* cells produce a galactose binding lectin called discoidin I (Simpson et al., 1974). Recent studies have implicated this protein in cell–substratum adhesion and ordered cell migration (Springer et al., 1984). Discoidin I is a homotetrameric protein whose complete primary sequence and subunit molecular weight (28 100) have been determined by molecular cloning and sequence analyses (Poole et al., 1981). Functional studies have revealed the existence of discrete cell binding and carbohydrate binding activities. The cell binding activity is associated with a tetrapeptide sequence homologous to the cell binding site of fibronectin (Pierschbacher et al., 1985). This sequence mediates cell–substratum adhesion by enabling discoidin I to bind to a specific receptor on the *D. discoideum* cell surface (Gabius et al., 1985). The function of the carbohydrate binding activity is presently unclear. Under certain conditions, it appears to mediate incorporation of intracellular discoidin I into multilamellar bodies and its subsequent externalization (Barondes et al., 1985; Cooper et al., 1986). Whether it also participates in the cellular aggregation process is unknown. Unlike the cell binding site, the carbohydrate binding site has yet to be identified within the primary sequence.

One approach to identification of the carbohydrate binding site is photoaffinity labeling, a method which has been applied to a variety of classes of binding proteins (Chowdhry & Westheimer, 1979; Bayley & Knowles, 1977; Ji, 1979). This strategy involves the use of an affinity ligand which contains a functional group that can be activated by ultraviolet (UV)[1] light to produce a reactive free radical. The photogenerated free radical, if situated at the binding site in a suitable orientation, is capable of forming covalent bonds with nearby amino acid residues, thereby irreversibly modifying that site. This approach has been applied to the identification and quantitation of particular binding proteins in complex mixtures, as well as to the identification of specific binding domains within purified proteins. Photoaffinity labeling has recently been applied to the study of carbohydrate binding proteins (Baenziger & Fiete, 1982; Bergey et al., 1986).

In this report, we describe the synthesis and characterization of a photoactivated affinity reagent for labeling the carbohydrate binding site of discoidin I. This probe, ^{125}I-labeled *N*-(4-azidosalicyl)galactosamine (^{125}I-GalNASA), interacts

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[1] Abbreviations: ASA, 4-azidosalicylic acid; ASF, asialofetuin; EDTA, ethylenediaminetetraacetic acid; EtNASA, *N*-(4-azidosalicyl)-ethanolamine; GalNAc, *N*-acetylgalactosamine; GalNASA, *N*-(4-azidosalicyl)galactosamine; $GalNH_2$, galactosamine; GlcNAc, *N*-acetylglucosamine; NHSASA, *N*-(hydroxysuccinimidyl)-4-azidosalicylic acid; PBS/Az, 20 mM potassium phosphate, pH 7.3, 150 mM NaCl, and 1 mM NaN_3; SDS, sodium dodecyl sulfate; 2-ME, 2-mercaptoethanol; UV, ultraviolet; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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with discoidin I in a manner suggestive of an association with the carbohydrate binding site. Photolysis induces a stable labeling of discoidin I, a large portion of which is specifically blocked by monosaccharide ligands and known inhibitors of discoidin I carbohydrate binding activity. Partial proteolytic digestion experiments have enabled us to identify a tryptic fragment of discoidin I whose photolabeling behavior closely resembles the carbohydrate binding behavior of the intact protein with respect to specificity and sensitivity of competition by monosaccharides. It is therefore likely that this fragment contains part or all of the carbohydrate binding site.

Materials and Methods

Materials. NHSASA was obtained from Pierce; carrier-free $Na^{125}I$ was from Amersham; *N*-(6-aminocaproyl)-galactosamine and ASF were obtained from Sigma. Unless indicated, all other reagents were purchased from commercial chemical suppliers and were of reagent grade or better.

Growth of Dictyostelium and Isolation of Discoidin I. Dictyostelium discoideum strain AX3 was grown axenically in the medium described by Loomis (1971) to a density of 1×10^7 cells/mL. Discoidin I was isolated and purified by affinity chromatography on Sepharose 4B to greater than 95% homogeneity as previously described (Frazier et al., 1975) and stored in 20 mM potassium phosphate, pH 7.3, 150 mM NaCl, and 1 mM NaN_3 (PBS/Az) containing 0.4 M galactose in aliquots at −70 °C.

^{125}I-Asialofetuin (^{125}I-ASF) Binding Assay. The ability of various ligands to bind to the carbohydrate binding site of discoidin I was assessed by their ability to compete against ^{125}I-ASF for binding to discoidin I immobilized on nitrocellulose, as previously described (Kohnken & Berger, 1987).

Preparation, Purification, and Radioiodination of ^{125}I-GalNASA. All procedures were performed under dim room light unless otherwise indicated (exposure of GalNASA to bright room light for 24 h resulted in less than 6% of maximal photolysis). Three 170-μL aliquots of freshly prepared 10 mg/mL *N*-(hydroxysuccinimidyl)-4-azidosalicylic acid (NHSASA) in acetone were added to 2.5 mL of 500 mM galactosamine ($GalNH_2$) in 0.1 M potassium phosphate, pH 7.3, at 10-min intervals. After a total of 30 min at 21 °C, the reaction mixture was diluted to 10 mL with 0.1 M NH_4HCO_3 and loaded onto a Sephadex G-10 column equilibrated in 0.1 M NH_4HCO_3. Fractions of 100 drops were collected, and after approximately 30 fractions (120 mL), the column was eluted with ethanol/water (1:1). GalNASA eluted after the included volume as a broad peak prior to ethanol/water elution (see Results). The appropriate fractions were pooled, lyophilized, resuspended in 1 mL of PBS/Az, and stored at −70 °C in 100-μL aliquots.

For iodination, 40 nmol of GalNASA was radioiodinated using Chloramine T (Greenwood et al., 1963). Iodination was terminated after 30 s with sodium metabisulfite, and the reaction mixture was loaded onto a 3-mL Sephadex G-10 column equilibrated in 0.1 M NH_4HCO_3. After 10–15 fractions (0.3 mL each) were collected, ^{125}I-GalNASA was eluted with ethanol/water (1:1) (see Results). The ^{125}I-GalNASA pool was dried under N_2, resuspended to 1 mL in PBS/Az, and stored in 50-μL aliquots at −70 °C.

Photolabeling of Discoidin I. To remove galactose, discoidin I was chromatographed on Bio-Gel P-6 equilibrated in PBS/Az. In a typical photolabeling reaction, 10 μg of protein was mixed with ^{125}I-GalNASA (approximately 4×10^5 cpm) in PBS/Az containing 1 mM $CaCl_2$. The reaction was performed in total volume of 50 μL in a microcentrifuge tube. Samples were cooled in an ice/water bath and photolyzed with a Minerallite UVG-11 for 3 min at a distance of 2.5 cm. Immediately after photolysis, protein and unreacted ^{125}I-GalNASA were separated by the addition of 450 μL of ice-cold acetone followed by a 5-min centrifugation. The protein pellet was resuspended in 50 μL of PBS/Az and reprecipitated with acetone. The pellet was again resuspended in PBS/Az, and sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2-ME) were added to 0.5% (w/v) each. The samples were boiled for 5 min and loaded onto an SDS–polyacrylamide slab gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R, destained, and dried. Incorporation of label into discoidin I was quantitated either by cutting out the corresponding band and counting in a γ counter or by densitometric scanning of autoradiograms using a Kontes fiber optic scanner interfaced with a Hewlett-Packard integrator.

Miscellaneous Assays. Protein was determined according to Lowry et al. (1951). SDS–polyacrylamide gel electrophoresis was performed on 16% slab gels as described by Laemmli (1970).

Table I: Competition of ^{125}I-ASF Binding to Discoidin I by Monosaccharides[a]

monosaccharide	rel binding (%)
none	100
galactose	24
$GalNH_2$	87
GalNAc	3
N-(6-aminocaproyl)-$GalNH_2$	31
GlcNAc	106

[a] ^{125}I-ASF binding to discoidin I was assayed as previously described (Kohnken & Berger, 1987) in the presence of the indicated monosaccharides at 50 mM. ^{125}I-ASF concentration was 1 nM at 5×10^5 cpm/pmol. A total of 8650 cpm was bound to 3 μg of discoidin I in the control sample (none) and was defined as 100% relative binding.

Results

Preparation and Characterization of GalNASA. With the goal of designing a photoaffinity probe for the carbohydrate binding site of discoidin I, we examined the ability of various monosaccharide derivatives of galactose to serve as ligands, using as an assay competition against ^{125}I-ASF binding to immobilized discoidin I (Kohnken & Berger, 1987). Table I shows that galactose was a more potent competitor than $GalNH_2$. Interestingly, acylation of the amino group of $GalNH_2$ greatly increased the ability to compete. *N*-Acetylgalactosamine (GalNAc) was a relatively potent competitor as expected (Frazier et al., 1975), inhibiting nearly completely at 50 mM. In contrast, *N*-acetylglucosamine (GlcNAc) was ineffective, consistent with previous reports (Frazier et al., 1975). Furthermore, relatively large acyl groups, such as 6-aminocaproyl, could still improve the competitive ability of the ligand relative to $GalNH_2$. On the basis of these results, we chose to prepare an acyl derivative of $GalNH_2$ which would be photoactivatable, capable of being radiolabeled, and small enough so as to react with the protein as close as possible to the carbohydrate binding site. The heterobifunctional reagent NHSASA reacts specifically with amino groups (Ji & Ji, 1982) and should, when coupled to $GalNH_2$, generate such a reagent. The reaction scheme for the synthesis of this probe, ^{125}I-GalNASA, is summarized in Figure 1.

We analyzed the reaction products of this synthetic scheme by several methods. In Figure 2, the generation of a new iodinatable product formed from the reaction between NHSASA and excess $GalNH_2$ is shown using thin-layer chromatography. When both NHSASA and $GalNH_2$ were present, and the reaction mixture subsequently iodinated, one

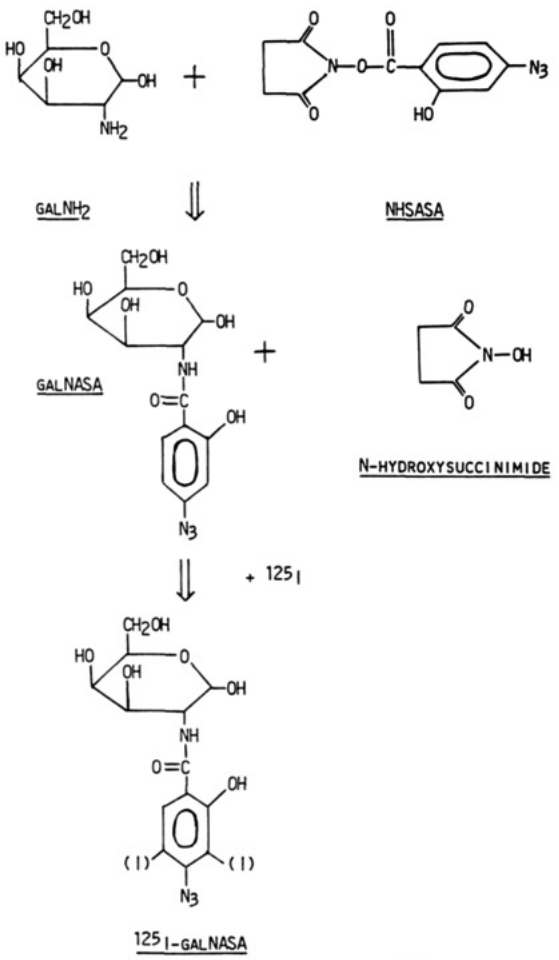

FIGURE 1: Reaction scheme for the synthesis of ^{125}I-GalNASA. The reaction conditions and procedures for purification and radioiodination of GalNASA are described under Materials and Methods.

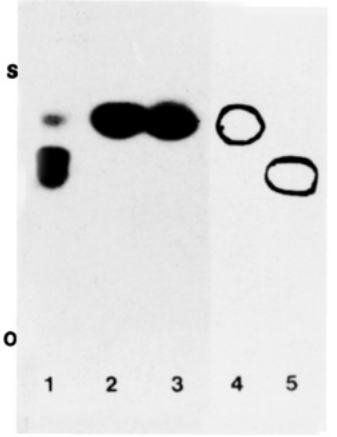

FIGURE 2: Thin-layer chromatography of products from the synthesis of ^{125}I-GalNASA. Eight microliters of 10 mg/mL NHSASA (290 nmol) was mixed with 192 μL of 500 mM $GalNH_2$ in 0.1 M potassium phosphate, pH 7.3, and incubated 30 min at 21 °C in the dark. Fifty-microliter aliquots from this reaction were iodinated by using $Na^{125}I$ (0.05 mCi, carrier free from Amersham) for 5 min using Chloramine T. Reactions were terminated with sodium metabisulfite, and 10 μL was spotted onto heat-activated silica gel G thin-layer plates (EM Science). Ascending chromatography was performed in 2-propanol/ammonium hydroxide/water (7:1.5:1.5), and spots were detected by autoradiography (lanes 1–3) or by visualization of UV-absorbing material under UV light (lanes 4 and 5). Lanes 1 and 5, complete reaction as described; lane 2, reaction as described except that NHSASA was omitted; lanes 3 and 4, reaction as described except that $GalNH_2$ was omitted. Circled spots in lanes 4 and 5 correspond to the migration behavior of material which absorbed UV light and underwent a color change in response to UV light; "o" and "s" refer to the origin and the solvent front of the chromatograph, respectively.

fast-migrating minor radioactive spot and a pair of slower migrating major radioactive spots were observed (lane 1). The slower of these two major spots corresponded to a UV-absorbing spot which bleached to a yellow color upon exposure to UV light (lane 5). When NHSASA was omitted, only a single rapidly migrating radioactive spot (lane 2), and no UV-absorbing spots, was observed. This presumably represented free ^{125}I. When $GalNH_2$ was omitted, again only a single fast-migrating radioactive spot was seen (lane 3) which now comigrated with a single UV-absorbing spot (lane 4). This radioactive spot therefore represented a mixture of ^{125}I-azidosalicylic acid (^{125}I-ASA) and free ^{125}I. [NHSASA rapidly hydrolyzes in aqueous solution to yield ASA and *N*-hydroxysuccinimide (Ji & Ji, 1982).] These data suggest that the slowly migrating pair of radioactive spots which occurred only when all reactants were present (lane 1) corresponded to ^{125}I-GalNASA. Support for this interpretation comes from additional experiments which are not shown. First, when a parallel reaction was performed with [^{3}H]$GalNH_2$, we observed a new tritiated species which comigrated with the UV-absorbing spot seen in lane 5; free [^{3}H]$GalNH_2$ remained at the origin. Second, we detected a decrease in the concentration of free amino groups upon reaction of $GalNH_2$ with excess NHSASA, as measured using trinitrobenzenesulfonate (Satake et al., 1960; Plapp et al., 1971), suggesting reaction of the amino group of $GalNH_2$ with NHSASA. We believe that the pair of radioactive spots seen in lane 1 represents the mono- and diiodinated forms of GalNASA. This interpretation was supported by the finding that further iodination of the reaction products seen in lane 1 with excess $K^{127}I$ caused the disappearance of the slower migrating member of the pair and a concomitant increase in the faster migrating member. Iodination with excess $K^{127}I$ also altered the behavior of the UV-absorbing material so that it now comigrated with the faster migrating radioactive spot.

Because we required both unlabeled and ^{125}I-GalNASA to be free of the other products and any contaminants, we chose to separate these reagents on a preparative scale. Sephadex G-10 was chosen not only for its gel filtration characteristics but also because it has been reported to bind aromatic compounds (Janson, 1967). During the course of fractionation, we observed surprising chromatographic behavior by GalNASA, ASA, and their iodinated derivatives. The profile of NHSASA, which had been hydrolyzed to free ASA and *N*-hydroxysuccinimide in buffer, is presented in Figure 3A. A peak of UV-absorbing material was observed in the included volume of the column (fractions 8–13), and upon washing the column with ethanol/water (1:1), a second peak was observed. Only the material in the second peak comigrated with ^{125}I-ASA on thin-layer plates and exhibited a change in its UV absorption spectrum characteristic of photosensitive reagents (data not shown), suggesting that this corresponded to free ASA. This UV absorption behavior of ASA, which was also observed for GalNASA, will be described below. The first peak presumably contained *N*-hydroxysuccinimide and any minor contaminants. When NHSASA was reacted with an excess of [^{3}H]$GalNH_2$ prior to chromatography, the profile in Figure 3B was observed. Again, a UV-absorbing peak was recovered in the included volume. Most of the radioactivity also eluted in this peak, and thin-layer chromatographic analysis suggested that this radioactivity represented unreacted [^{3}H]$GalNH_2$ (data not shown). A second peak, which eluted after the included volume but prior to elution with ethanol/water, also contained both radioactivity and UV-absorbing material. This radioactivity migrated on thin-layer plates at

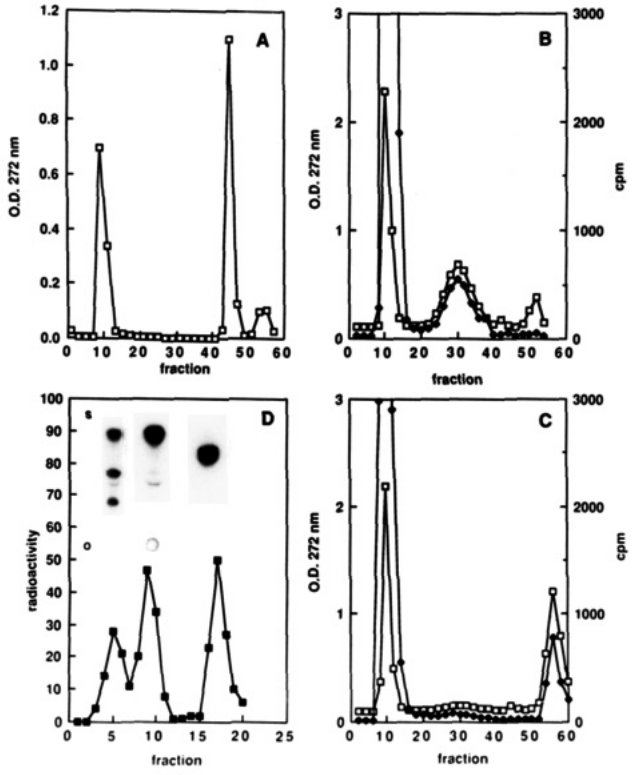

FIGURE 3: Sephadex G-10 chromatography of products from the synthesis of GalNASA and its derivatives. (A) Ten microliters of 10 mg/mL NHSASA was incubated with 20 μL of 0.1 M potassium phosphate, pH 7.3, for 30 min, diluted to 1 mL with 0.1 M NH_4HCO_3, and applied to a 12-mL Sephadex G-10 column equilibrated in 0.1 M NH_4HCO_3. After collection of 33 fractions eluted with NH_4HCO_3 (about 33 mL), the column was further eluted with ethanol/water (1:1). (B) Ten microliters of 10 mg/mL NHSASA was mixed with 20 μL of 500 mM $GalNH_2$ in 0.1 M potassium phosphate, pH 7.3, plus 10 μL of [^{3}H]$GalNH_2$ (10 μCi). The reaction proceeded for 30 min at 21 °C and was diluted to 1 mL with 0.1 M NH_4HCO_3, and chromatography was performed as described in (A). (C) Reaction of NHSASA, $GalNH_2$, and [^{3}H]$GalNH_2$ was performed as described in (B). After the reaction had proceeded for 30 min, 1 μL of 500 mM KI and 20 μL of 5 mg/mL Chloramine T were added to the incubation. After 1 min, iodination was terminated with 20 μL of 10 mg/mL sodium metabisulfite. The mixture was then diluted, and chromatography was performed as described in (A). (D) Four microliters of 10 mg/mL NHSASA was mixed with 96 μL of 500 mM $GalNH_2$ in 0.1 M potassium phosphate, pH 7.3, for 30 min. A 50-μL aliquot was labeled with 1 mCi of Na^{125}I for 30 s with Chloramine T. Upon termination, the iodination reaction mixture was applied to a 3-mL Sephadex G-10 column equilibrated in PBS/Az. The column was eluted with PBS/Az and after 12 fractions (about 10 mL) further eluted with ethanol/water (1:1). Inset: Autoradiogram of pooled fractions from this column which had been chromatographed on thin-layer plates as described in Figure 1; "o" and "s" refer to the origin and solvent front of the chromatograph, respectively. (□) Absorbance at 272 nm; (♦) cpm of ^{3}H per fraction; (■) ^{125}I detected by a γ scintillator at a distance of 12 cm expressed in arbitrary units.

the same location as the slower member of the pair of ^{125}I-GalNASA spots (Figure 2, lanes 1 and 5). Uniodinated GalNASA also migrates at this location (data not shown), suggesting that this peak consists of [^{3}H]GalNASA. After elution with ethanol/water, a peak was again observed which had UV absorbance, but no radioactivity, representing ASA which had not reacted with $GalNH_2$. These results demonstrate that Sephadex G-10 effectively resolved GalNASA from the major reactants and side products of its synthesis.

Iodination caused a marked change in the Sephadex G-10 chromatographic behavior of both GalNASA and free ASA. When the reaction mixture described in Figure 3B was iodinated with an excess of K^{127}I and then fractionated, the profile in Figure 3C was observed. The included volume peak remained as in Figure 3B, but no peak was observed where GalNASA had previously eluted. Instead, a UV-absorbing and radioactive peak was obtained only after elution with ethanol/water. This material comigrated on thin-layer plates with the faster migrating component of the pair of ^{125}I-GalNASA spots seen in Figure 2, lane 1. Thus, iodination increased the retention of GalNASA on Sephadex G-10 so that it eluted only after washing with ethanol/water. Surprisingly, iodination had the reverse effect on ASA, causing it to elute from Sephadex G-10 prior to ethanol/water washing. This was demonstrated by the experiment shown in Figure 3D in which a GalNASA reaction mixture, similar to that described for Figure 3B, was iodinated with Na^{125}I. In this case, the relative quantities of reagents and the dimensions of the Sephadex column were different from the experiments in Figure 3A–C (see figure legend). The first peak of radioactivity contained free ^{125}I, as shown by the thin-layer chromatograph inset, plus unidentified contaminants. The second peak, which was retained on the column beyond the included volume, consisted mostly of ^{125}I-ASA, on the basis of its UV spectrum and thin-layer chromatographic behavior. The last peak, which was eluted from the column with ethanol/water, contained the pair of slowly migrating spots indicative of ^{125}I-GalNASA, as seen in the thin-layer chromatograph. On the basis of these results, we chose as our standard procedure to prepare uniodinated GalNASA by a regimen analogous to that in Figure 3B. The fractions from the second UV-absorbing peak of the Sephadex G-10 column were pooled and used as purified GalNASA. To prepare ^{125}I-GalNASA, an aliquot of this pool was radioiodinated and refractionated on Sephadex G-10, and the ^{125}I peak eluting after ethanol/water was collected.

Both ASA and GalNASA have an absorbance maximum at 272 nm in PBS/Az with a shoulder or broad peak at 310–330 nm, similar to that reported for *N*-(4-azidosalicyl)-6-amido-6-deoxyglucopyranose (Shanahan et al., 1985). Upon photolysis, the absorbance at 272 nm decreased, and the solution became slightly yellow, serving as a diagnostic test for the presence of ASA or GalNASA in the solution. This same bleaching behavior also occurred on thin-layer plates after exposure to UV light, aiding in identification of those compounds. The UV-absorbing contaminants observed in the first peaks of the Sephadex G-10 columns in Figure 3 had an absorbance maximum at 265 nm and did not exhibit bleaching due to UV exposure. Using [^{3}H]$GalNH_2$ with a known specific activity, we were able to estimate the extinction coefficient of GalNASA at 272 nm to be 1.5×10^4 M^{-1} cm^{-1}. This is in agreement with extinction coefficients reported for other aromatic azides (Ji, 1983).

GalNASA Competition for the Carbohydrate Binding Site of Discoidin I. In order to use GalNASA as a probe of the carbohydrate binding site of discoidin I, it was essential to demonstrate that it behaved as a ligand. In an earlier report, we observed that known ligands of discoidin I were competitors of ^{125}I-ASF binding to discoidin I (Kohnken & Berger, 1987). We therefore examined the ability of GalNASA to compete for discoidin I in the ^{125}I-ASF binding assay. The results are shown in Figure 4. We observed that GalNASA was an effective competitor, comparable to GalNAc, with a $K_{i,app}$ of 0.8 mM. Surprisingly, photolysis of GalNASA prior to inclusion in the ^{125}I-ASF binding assay increased its ability to compete, reducing the $K_{i,app}$ to approximately 40 μM (Figure 4A). This was not due to the photolyzed ASA moiety acting as an inhibitor of discoidin I carbohydrate binding; as shown in Figure 4B, *N*-(4-azidosalicyl)ethanolamine (EtNASA) was

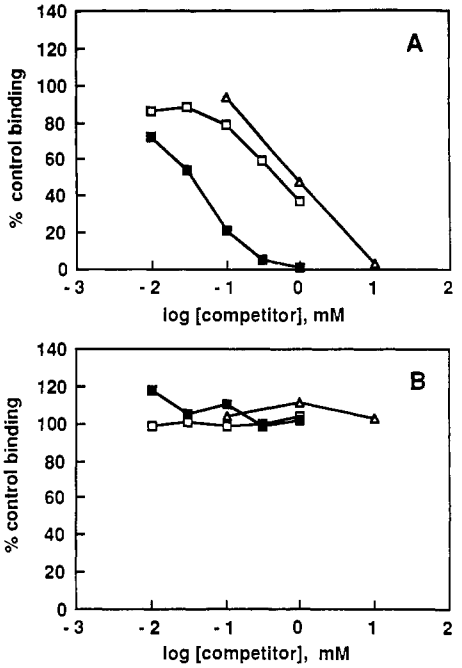

FIGURE 4: Competition by GalNASA in the ^{125}I-ASF binding assay. Discoidin I was assayed for binding of ^{125}I-ASF as described previously (Kohnken & Berger, 1987). EtNASA was prepared and purified as described for GalNASA under Materials and Methods. In cases where UV photolysis was performed, samples were irradiated at a distance of 4 cm with a germicidal lamp at 4 °C for 5 min with shaking in 0.5 mL of 1 mM $CaCl_2$ and PBS/Az. Data presented are the average from two separate experiments; 7876 cpm of ^{125}I-ASF was bound to discoidin I in the absence of competitors and was defined as 100% control binding. (A) Competition by GalNAc (△) and by GalNASA with (■) and without (□) prior UV photolysis. (B) Competition by ethanolamine (△) and by EtNASA with (■) and without (□) prior UV photolysis.

not inhibitory, and prior photolysis had no effect.

UV-Dependent Labeling of Discoidin I with ^{125}I-GalNASA. We next examined the ability of ^{125}I-GalNASA to photochemically label discoidin I. The extent of labeling was quantitated by measuring the incorporation of ^{125}I into the discoidin I band after electrophoresis in SDS–polyacrylamide gels as described under Materials and Methods. During a 5-min time course, labeling of discoidin I asymptotically approached a maximum. No labeling was detected in the absence of UV light (not shown). Under similar conditions but in the absence of protein, 100 μM GalNASA was photolyzed with a similar time course, as determined by the change in absorbance at 272 nm (not shown). Thus, the saturation of labeling presumably reflected the completion of photolysis. Since labeling persisted after the denaturing conditions associated with SDS gel electrophoresis, we assumed that it represented a covalent association between discoidin I and ^{125}I-GalNASA. Exposure of discoidin I to UV light in the absence of GalNASA had no effect on its subsequent ability to bind ^{125}I-ASF, indicating that UV treatment per se did not adversely affect carbohydrate binding activity (not shown).

The labeling described above could represent specific reaction of ^{125}I-GalNASA with the carbohydrate binding site and/or carbohydrate independent reactivity with protein. To evaluate the contributions of these two potential forms of labeling, we examined the effects on photolabeling of known ligands or inhibitors of discoidin I carbohydrate binding activity. As a control, we also tested the effects of these reagents on the photolabeling of bovine serum albumin by ^{125}I-GalNASA. The results are summarized in Table II. At concentrations which inhibited ^{125}I-ASF binding to discoidin I by

Table II: Photolabeling of Discoidin I and Bovine Serum Albumin with ^{125}I-GalNASA[a]

additions	rel incorpn of radioact. (%)	
	discoidin I	bovine serum albumin
none	100	100
GalNAc (60 mM)	52	119
ASF (0.12 mg/mL)	60	113
GalNASA (100 μM)	40	108
GlcNAc (60 mM)	91	119
EDTA (2 mM)	56	118

[a] Photolabeling was performed and quantitated as described under Materials and Methods. Results for each protein are expressed as a percentage of labeling which occurred in the absence of any additions. A total of 11 828 cpm were incorporated into discoidin I and 38 340 cpm into bovine serum albumin in the absence of any competitors.

90% or more (Kohnken & Berger, 1987; Figure 4A), GalNAc and ASF inhibited ^{125}I-GalNASA photolabeling of discoidin I by 40–50%. GalNASA at 100 μM, which reduced ^{125}I-ASF binding by 80% (Figure 4A), reduced photolabeling of discoidin I by 60%. GlcNAc, which is not a competitor of carbohydrate binding by discoidin I, had little effect on photolabeling. The divalent cation chelator EDTA, which is known to block carbohydrate binding activity of discoidin I (Alexander et al., 1979; Cooper et al., 1983), also inhibited photolabeling by 44%. In contrast, none of these reagents had any effect on photolabeling of bovine serum albumin at the concentrations shown. These data support the conclusion that about half of the photolabeling of discoidin I by ^{125}I-GalNASA arose from an interaction of GalNASA at the carbohydrate binding site. This component of photolabeling will be referred to as GalNAc-sensitive labeling. The other half of discoidin I photolabeling occurred via a carbohydrate-insensitive process.

We next performed a number of experiments to analyze the process by which covalent attachment of ^{125}I-GalNASA to discoidin I occurred. True photoaffinity labeling, unlike a related process called pseudophotoaffinity labeling, should be insensitive to free radical scavengers, which cannot gain access to the activated ligand while it is in the binding site (Ruoho et al., 1973). These reagents can thus help distinguish between these two processes. We found that potential scavengers such as Tris, butylated hydroxytoluene, and *p*-aminobenzoic acid had little effect (less than 20% inhibition) on total labeling of discoidin I at concentrations which did not interfere by absorption of UV light. Another scavenger, 2-ME, was a potent inhibitor such that at 1 mM, up to 90% of total labeling was prevented. However, this inhibition could not be attributed solely to a scavenging effect, since 2-ME also blocked carbohydrate binding to discoidin I (58% at 1 mM) in the ^{125}I-ASF binding assay. Thus, it was difficult to interpret these results unequivocally. Nevertheless, these data raised the possibility that labeling of discoidin I by ^{125}I-GalNASA did not occur entirely by true photoaffinity labeling.

Another way to analyze the mechanism of labeling was to examine the ability of prephotolyzed ^{125}I-GalNASA to label discoidin I. Label incorporation from prephotolyzed probe would reflect the involvement of long-lived photogenerated products. This phenomenon has been reported to occur using *N*-(4-azido-2-nitrophenyl)taurine to label rhodopsin (Mas et al., 1979). Incubation of discoidin I with prephotolyzed ^{125}I-GalNASA resulted in incorporation of label which was stable to SDS gel electrophoresis. Under comparable conditions (same duration of photolysis, same duration of exposure of discoidin I to photolyzed ^{125}I-GalNASA), the total level of labeling with prephotolyzed ^{125}I-GalNASA was 46% that of the incorporation observed when photolysis of ^{125}I-GalNASA was performed in the presence of discoidin I. When inhibition

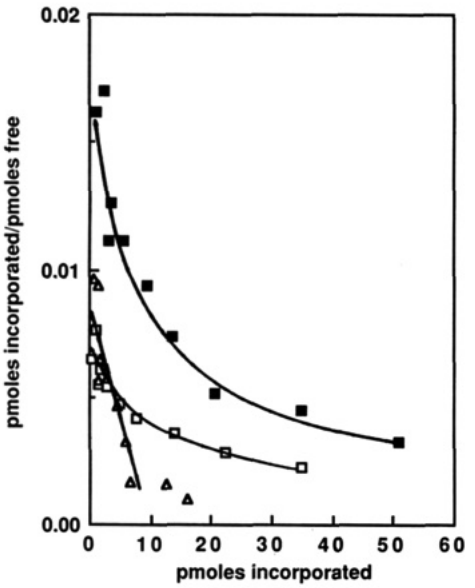

FIGURE 5: GalNASA concentration dependence of discoidin I photolabeling. Discoidin I (10 μg) was photolabeled as described under Materials and Methods except that increasing quantities of unlabeled GalNASA were included in the reaction. Label incorporation was quantitated as described under Materials and Methods. Picomoles of GalNASA incorporated and incorporated:free ratios were calculated and plotted according to Scatchard (1949). (■) Labeling in the absence of GalNAc. (□) Labeling in the presence of 100 mM GalNAc. (△) GalNAc-sensitive labeling (defined as the calculated difference between the previous two conditions). Radioactivity incorporated into discoidin I in the absence of GalNAc and unlabeled GalNASA was 9046 cpm.

by GalNAc was examined for both of these conditions, labeling of discoidin I with prephotolyzed ^{125}I-GalNASA was inhibited 30% as opposed to 59% when photolysis was performed in the presence of discoidin I. These data argue that at least some GalNAc-sensitive labeling could occur due to long-lived, photogenerated products and therefore does not represent a true photoaffinity process. Nevertheless, the existence of a GalNAc-sensitive component of labeling is consistent with an affinity labeling interaction at the carbohydrate binding site.

GalNASA Concentration Dependence of Labeling of Discoidin I. As one way of confirming that this labeling was representative of affinity labeling, we examined whether GalNAc-sensitive labeling occurred via an interaction at a single site. The dependence of discoidin I labeling on GalNASA concentration in the presence and absence of GalNAc is presented in Figure 5. GalNASA concentration was varied from 1 to 320 μM in this experiment, and the data were plotted according to Scatchard (1949). Control experiments indicated that GalNASA was nearly completely photolyzed over the entire concentration range examined in this experiment (data not shown); therefore, the outcome should not have been influenced by this potential artifact. Total labeling exhibited complex behavior, with a concave-upward plot indicative of more than one type of labeling interaction (as expected from the data in Table II). Labeling in the presence of GalNAc (GalNAc insensitive) was also complex. However, the calculated difference between the two curves, representing GalNAc-sensitive labeling, behaved largely as a single population of sites. The efficiency of labeling at this site, defined as the percent of sites labeled when binding is saturating and assuming one site per discoidin I monomer, was approximately 2.5%. In several experiments, the K_d estimated from the slope of the GalNAc-sensitive curve was 15–40 μM, in good agreement with the $K_{i,app}$ of photolyzed GalNASA for inhibition of ^{125}I-ASF binding (40 μM, Figure 4A). It therefore seems likely that GalNAc-sensitive labeling occurred via interaction at a single set of sites with an affinity for photolyzed GalNASA similar to that of the carbohydrate binding site. It should be noted that in addition to this form of GalNAc-sensitive labeling, we consistently observed a lower affinity component of GalNAc-sensitive labeling of discoidin I (Figure 5). The significance of this behavior is unclear.

Localization of Labeling on Proteolytic Fragments of Discoidin I. If GalNAc-sensitive labeling, or some portion of it, represents affinity labeling of the carbohydrate binding site, than it should be localizable to distinct fragments of discoidin I. Furthermore, labeling of such fragments should exhibit a sensitivity to competitors identical with that of carbohydrate binding activity. Therefore, we examined whether GalNAc-sensitive labeling could be localized to specific fragments of discoidin I. In the experiments shown in Figure 6, discoidin I was labeled with ^{125}I-GalNASA in the absence and presence of 50 mM GalNAc and then subjected

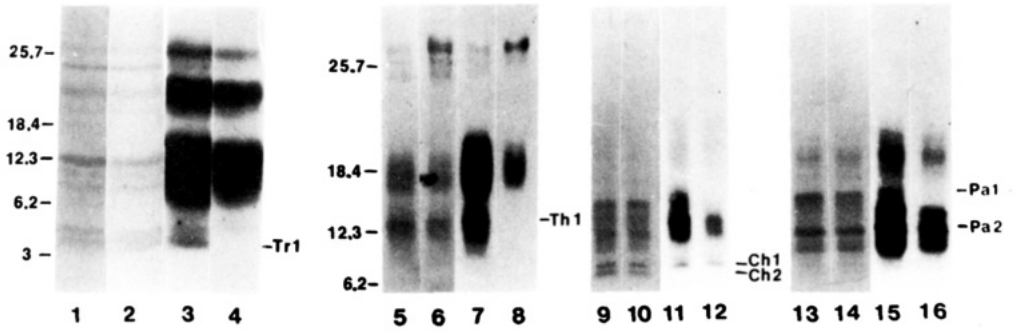

FIGURE 6: Distribution of photolabel incorporation into proteolytic fragments of discoidin I. Discoidin I was photolabeled with ^{125}I-GalNASA as described under Materials and Methods in the absence (odd-numbered lanes) and the presence (even-numbered lanes) of 50 mM GalNAc. Following acetone precipitation, discoidin I was resuspended in 40 μL of the indicated proteolysis buffer and treated as follows: (Lanes 1–4) 1 mM $CaCl_2$, 0.18% SDS (w/v), and 0.18% 2-ME (w/v) in PBS/Az, boiled for 2 min, cooled to 21 °C, and incubated with 3 μg of trypsin added in an additional 40 μL of PBS/Az for 10 min; (lanes 5–8) same as lanes 1–4 except that 0.3 μg of thermolysin in 40 μL was added for proteolysis; (lanes 9–12) 10 mM $CaCl_2$, 0.18% SDS (w/v), and 0.18% 2-ME (w/v) in 100 mM Tris-HCl, pH 7.6, boiled for 2 min, cooled to 21 °C, and incubated with 0.1 μg of chymotrypsin in an additional 40 μL of 10 mM $CaCl_2$ in 100 mM Tris-HCl, pH 7.6, for 10 min; (lanes 13–16) 5 mM cysteine, 1 mM EDTA in PBS/Az, and 0.1 μg of papain added in an additional 40 μL of the same buffer and incubated for 10 min. Proteolysis was terminated by adding SDS and 2-ME each to 0.5% (w/v) and boiling for 5 min. Samples were analyzed by SDS gel electrophoresis on 16% polyacrylamide slab gels. Stained gel lanes and their corresponding autoradiograms were scanned as described under Materials and Methods. In some cases, a shorter exposure autoradiogram was scanned. The first two lanes in each group of four are the Coomassie blue stained gels, and the second two lanes are the corresponding autoradiograms. Indicated molecular weights, expressed × 10^{-3}, are based on the migration of BRL low molecular weight standards. The locations of protein bands described in the text are specifically designated.

to partial digestion with several proteases. The samples were analyzed by SDS gel electrophoresis, and the labeling patterns with and without GalNAc were compared. At the outset, several predictions could be made assuming that GalNAc-sensitive labeling exhibited a restricted localization. First, fragments containing the GalNAc-sensitive labeling site should display increasing GalNAc inhibition of label incorporation with decreasing size (as GalNAc-insensitive labeling is partitioned onto other fragments). Second, labeling of fragments from regions not containing the carbohydrate-sensitive site should be resistant to GalNAc. Finally, it is also possible that some fragments might be labeled to an insignificant extent in the absence or presence of GalNAc. The digests shown in Figure 6 contain examples of each of these type fragments.

Due to some variability in the degree of digestion between companion samples, it was essential to normalize the extent of labeling of a particular fragment to the amount of that fragment, in order to assess the GalNAc sensitivity of any given band. We therefore quantitated both the Coomassie blue bands and the autoradiographic bands by scanning densitometry. For the experiment shown, labeling of intact discoidin I was inhibited 51% by GalNAc, and most labeled bands exhibited between 40% and 60% inhibition. Bands which exhibited a high degree of GalNAc inhibition were seen in both trypsin (lanes 1–4) and thermolysin (lanes 5–8) digests. Band Tr1 in the trypsin digest was inhibited by 98% by GalNAc, while band Th1 in the thermolysin digest was inhibited 92%. On the other hand, bands Ch1 in the chymotrypsin (lanes 9–12) and Pa2 in the papain (lanes 13–16) digests were inhibited to a lesser degree than most other bands. Ch1 was inhibited 32%, and Pa2 was inhibited only 22% by GalNAc. Finally, bands Pa1 in the papain digest and Ch2 in the chymotrypsin digest exhibited very low levels of labeling in the absence or presence of GalNAc (Pa1 was sensitive to GalNAc, but the point addressed here is that its level of labeling was quite low relative to the amount of protein in the band). These bands were not derived from the proteases themselves since their appearance on SDS gels depended upon inclusion of discoidin I in the incubation (data not shown). These data indicate that GalNAc-sensitive labeling could be localized to discrete fragments of discoidin I.

Although we observed other discoidin I fragments which exhibited strong GalNAc inhibition, Tr1 appeared in the partial tryptic digests most consistently. We therefore examined in more detail the labeling behavior of Tr1. Figure 7A shows the specificity of sugar inhibition of photolabeling. Labeling of Tr1 (lane 1) was completely abolished by 50 mM GalNAc (lane 2) but was unaffected by the same concentration of GlcNAc (lane 3). This specificity coincides with the known carbohydrate specificity of discoidin I. In Figure 7B, we examined the concentration dependence of GalNAc inhibition of Tr1 photolabeling. Labeling of Tr1 was more sensitive to GalNAc competition than other labeled tryptic fragments, with 50% inhibition occurring at approximately 1 mM (lane 3). This value agrees well with the concentration dependence of GalNAc inhibition of ^{125}I-ASF binding to discoidin I (see Figure 4A). These data argue that labeling of Tr1 resulted from an interaction of ^{125}I-GalNASA at the carbohydrate binding site of discoidin I and that Tr1 probably represents some portion of that site.

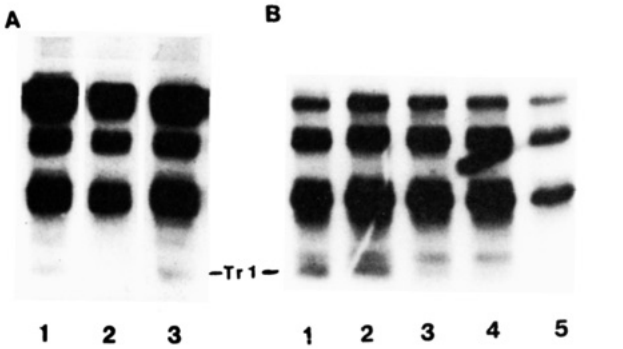

FIGURE 7: Photolabeling behavior of the tryptic peptide Tr1 derived from discoidin I. Discoidin I was photolabeled in the presence and amounts of sugars indicated below and acetone precipitated as described under Materials and Methods. Samples were digested with trypsin in 0.03% SDS (w/v), 0.03% 2-ME (w/v), and 1 mM $CaCl_2$ in PBS/Az at a trypsin:discoidin I mass ratio of 0.7:10 for 10 min at 21 °C. Digestion was terminated by the addition of SDS and 2-ME (0.5% w/v each) and boiled for 5 min. Samples were analyzed on 16% polyacrylamide SDS gels, and autoradiograms of those gels are presented. Coomassie blue staining patterns are not shown, but within each experiment, there was little variability among samples. (A) Specificity of sugar inhibition. During photolabeling, samples contained no competitor (lane 1), 50 mM GalNAc (lane 2), or 50 mM GlcNAc (lane 3). (B) Concentration dependence of GalNAc inhibition of photolabeling. GalNAc concentration during photolabeling was varied as follows: lane 1, 0 mM; lane 2, 0.1 mM; lane 3, 1 mM; lane 4, 10 mM; lane 5, 50 mM.

Discussion

Photochemical labeling has been a valuable technique in the identification of binding proteins and binding domains within proteins. A number of recent studies have been directed at carbohydrate binding proteins. Baenziger and Fiete (1982) described photochemical labeling of both *Ricinus communis* agglutinin and concanavalin A using glycopeptides derivatized with an azidonitrophenyl compound. Bergey et al. (1986) prepared a salivary glycoprotein coupled to ^{125}I-ASA to examine its interaction with bacterial components. Both of these studies used ligands which exhibited high-affinity interactions ($K_d < 10^{-6}$ M) with the corresponding binding sites, so as to maximize the specificity of the photochemical labeling reactions (Bayley & Knowles, 1977). However, in each of these cases, the photoactive group was situated at a considerable distance from the ligand moieties, and therefore, covalent attachment of the ligand potentially occurred at some distance from the protein residues actually involved in binding. Attempts to use small probes to overcome this obstacle often suffer from low affinity between the probe and its binding site. The higher concentrations of probe required in these studies frequently result in high levels of nonspecific labeling. In spite of this limitation, photochemical derivatives of monosaccharides have been used successfully to study concanavalin A (Beppu et al., 1975), glyceraldehyde-3-phosphate dehydrogenase (May, 1986), and the glucose transporter of erythrocyte membranes (Shanahan et al., 1985). Recently, Lee and Lee (1986) prepared a glycopeptide in which the photochemical moiety was coupled to the terminal sugar residues involved in high-affinity binding to the Gal/GalNAc receptor of mammalian liver cells, thus overcoming the weaknessess of both earlier approaches.

GalNASA was chosen for the purpose of identifying the carbohydrate binding site of discoidin I for several reasons. First, acylation of $GalNH_2$ enhanced its ability to compete for the carbohydrate binding site of discoidin I in the ^{125}I-ASF binding assay, suggesting that GalNASA might behave as a ligand with suitable affinity for discoidin I. Second, GalNASA could be labeled to a high specific activity with ^{125}I so that low concentrations could be used to minimize nonspecific labeling. Finally, GalNASA is a small and reactive reagent which should maximize the likelihood of covalent attachment in the immediate neighborhood of the carbohydrate binding site.

The structure of GalNASA was confirmed by a number of observations. First, a new UV-absorbing material was observed upon reactions of $GalNH_2$ and NHSASA. Second, this material contained a $GalNH_2$ moiety, since tritium label comigrated with it when the reaction was performed with [^{3}H]$GalNH_2$, both on thin-layer plates and on Sephadex G-10 chromatography. Third, it contained the ASA moiety, based on its UV spectral characteristics and the ability to be iodinated. Finally, reaction of $GalNH_2$ with NHSASA resulted in a decreased number of free amino groups, suggesting that an amide linkage was formed between the two reactants.

When we examined the ability of GalNASA to compete for discoidin I in the ^{125}I-ASF binding assay, we observed that it was indeed a competitor for the carbohydrate binding site, comparable in affinity to GalNAc. Furthermore, its ability to compete was enhanced about 20-fold ($K_{i,app}$ of 800–40 μM) by UV photolysis. This behavior was unique to GalNASA in that EtNASA did not compete whether or not it was prephotolyzed. These data imply that GalNASA and photolyzed GalNASA behaved as ligands for the carbohydrate binding site of discoidin I.

Labeling of discoidin I with ^{125}I-GalNASA was entirely dependent upon and apparently proportional to photolysis with UV light. Some labeling was also observed when ^{125}I-GalNASA was photolyzed prior to addition of discoidin I. This suggested that a portion of the labeling of discoidin I was not true photoaffinity labeling but rather occurred via long-lived, photogenerated products of GalNASA. Photoaffinity labeling refers specifically to a process in which the photosensitive ligand becomes activated and reacts covalently at its binding site before it can dissociate from that site. A related phenomenon has been described, called pseudophotoaffinity labeling (Ruoho et al., 1973), in which the activated ligand can dissociate prior to covalent attachment. In this situation, any photoactivated ligand in the reaction mixture can couple to the protein, either specifically at the binding site or nonspecifically at other sites on the protein. Pseudophotoaffinity labeling, therefore, is analogous to affinity labeling.

Regardless of the precise mechanism of labeling, several lines of evidence argue that a portion of discoidin I labeling by ^{125}I-GalNASA represents an affinity labeling reaction at the carbohydrate binding site and that this labeling can be used to identify proteolytic fragments derived from that site. First, GalNASA behaved as a ligand for the carbohydrate binding site as discussed above. Second, approximately 50% of discoidin I labeling by ^{125}I-GalNASA exhibited a sensitivity to known inhibitors of carbohydrate binding by discoidin I. Labeling was inhibited comparably by GalNAc, ASF, and EDTA at concentrations which entirely blocked ^{125}I-ASF binding to discoidin I (Kohnken & Berger, 1987). On the other hand, labeling was completely insensitive to GlcNAc, consistent with the failure of this sugar to compete in the ^{125}I-ASF binding assay. Labeling of bovine serum albumin, which does not specifically interact with carbohydrates, and indeed binds ^{125}I-ASF very poorly (Kohnken & Berger, 1987), showed no inhibition by sugars or EDTA. These data suggest that a portion (40–60%) of discoidin I labeling by ^{125}I-GalNASA exhibited behavior consistent with an interaction of the probe at the carbohydrate binding site. Third, the concentration at which GalNASA inhibited ^{125}I-ASF binding to discoidin I ($K_{i,app}$ of approximately 40 μM for prephotolyzed GalNASA) was in good agreement with the dependence of the GalNAc-sensitive component of photolabeling on ^{125}I-GalNASA concentration (K_d of 15–40 μM), suggesting that labeling occurred at the ligand binding site. Finally, proteolytic digestion studies indicated that a GalNAc-sensitive component of photolabeling could be localized to discrete, small fragments of discoidin I, suggesting a specific site of labeling rather than a random distribution.

Although many fragments exhibited some degree of GalNAc-sensitive labeling, fragment Tr1, generated by partial trypsin digestion, appeared a most promising candidate for some portion of the carbohydrate binding site of discoidin I. Its labeling by ^{125}I-GalNASA was entirely inhibited by GalNAc and was insensitive to GlcNAc. Furthermore, the concentration dependence of GalNAc inhibition of labeling was similar to the concentration dependence of GalNAc inhibition of ^{125}I-ASF binding to discoidin I (K_i of approximately 1 mM in both cases). These data argue that the specificity and sensitivity of Tr1 labeling closely paralleled those of carbohydrate binding activity. Tr1 thus probably represents a portion of the carbohydrate binding site.

Not all GalNAc-sensitive labeling observed in the tryptic digests was localized to Tr1. This could be due to several factors. First, it should be noted that Tr1 is a product of partial protelytic digestion, and its detection depended on choosing reaction conditions which counterbalanced its generation and subsequent degradation. Thus, in the digests shown, a considerable fraction of the GalNAc-sensitive labeling appeared in larger fragments which presumably contained all or part of the Tr1 sequence. Second, Tr1 may represent only a portion of the carbohydrate binding site and would thus not be expected to contain all of the GalNAc-sensitive labeling. It should be noted that for several carbohydrate binding proteins, the binding sites involve residues from more than one contiguous region of the primary amino acid sequence (Quiocho, 1986). We cannot exclude this possibility for discoidin I. Third, although GalNAc-sensitive labeling apparently arose from an interaction of ^{125}I-GalNASA at the carbohydrate binding site, the actual location of some of that labeling may have been at some nearby, kinetically favorable site(s) on the protein. This would probably involve long-lived photogenerated reactants. We feel that Tr1 labeling is probably not a reflection of such a process for the following reasons: (a) its relatively low level of labeling, indicating that it is probably not inherently a highly reactive site; (b) its complete inhibition by GalNAc, which would be unlikely for a site which is at some distance from the carbohydrate binding site and therefore accessible for labeling by long-lived photogenerated reactants in the surrounding medium.

We are currently attempting to isolate Tr1 for sequence analysis, which will allow its localization within the known primary sequence of discoidin I (Poole et al., 1981). Corroboration of the involvement of this fragment in the carbohydrate binding site by other methods, such as labeling of a sulfhydryl which is required for ^{125}I-ASF binding (Kohnken & Berger, 1987) or the use of monoclonal antibodies which interfere with carbohydrate binding, is also in progress. This information will provide a framework for addressing the role of the carbohydrate binding activity of discoidin I in cell–substratum adhesion and other developmental processes of *Dictyostelium discoideum*.

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REFERENCES

Alexander, S., Cibulsky, S. M., & Lerner, R. A. (1983) *Differentiation (Berlin) 24*, 209–212.

Baenziger, J. U., & Fiete, D. (1982) *J. Biol. Chem. 257*, 4421–4425.
Barondes, S. H., Haywood-Reid, P. L., & Cooper, D. N. W. (1985) *J. Cell Biol. 100*, 1825–1833.
Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.
Beppu, M., Terao, T., & Osawa, T. (1975) *J. Biochem. (Tokyo) 78*, 1013–1019.
Bergey, E. J., Levine, M. J., Reddy, M. S., Bradway, S. D., & Al-Hashimi, I. (1986) *Biochem. J. 234*, 43–48.
Chowdhry, V., & Westheimer, F. H. (1979) *Annu. Rev. Biochem. 48*, 293–325.
Cooper, D. N., Lee, S.-C., & Barondes, S. H. (1983) *J. Biol. Chem. 258*, 8745–8750.
Cooper, D. N. W., Haywood-Reid, P. L., Springer, W. R., & Barondes, S. H. (1986) *Dev. Biol. 114*, 416–425.
Erdos, G. W., & Whitaker, D. (1983) *J. Cell Biol. 97*, 993–1000.
Frazier, W. A., Rosen, S. D., Reitherman, R. W., & Barondes, S. H. (1975) *J. Biol. Chem. 250*, 7714–7721.
Gabius, H.-J., Springer, W. R., & Barondes, S. H. (1985) *Cell (Cambridge, Mass.) 42*, 449–456.
Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.
Janson, J.-C. (1967) *J. Chromatogr. 28*, 12–20.
Ji, T. H. (1979) *Biochim. Biophys. Acta 559*, 39–69.
Ji, T. H. (1983) *Methods Enzymol. 91*, 580–609.
Ji, T. H., & Ji, I. (1982) *Anal. Biochem. 121*, 286–289.
Kohnken, R. E., & Berger, E. A. (1987) *Biochemistry 26*, 3949–3957.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, R. T., & Lee, Y. C. (1986) *Biochemistry 25*, 6835–6841.
Loomis, W. F., Jr. (1971) *Exp. Cell Res. 64*, 484–486.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mas, M. T., Wang, J. K., & Hargrave, P. A. (1980) *Biochemistry 19*, 684–692.
May, J. M. (1986) *J. Biol. Chem. 261*, 2542–2547.
Pascual, A., Casanova, J., & Samuels, H. H. (1982) *J. Biol. Chem. 257*, 9640–9647.
Pfeuffer, T. (1977) *J. Biol. Chem. 252*, 7224–7234.
Pierschbacher, M. D., Hayman, E. G., & Ruoslahti, E. (1985) *J. Cell. Biochem. 28*, 115–126.
Plapp, B. V., Moore, S., & Stein, W. H. (1971) *J. Biol. Chem. 246*, 939–945.
Poole, S., Firtel, R. A., Lamar, E., & Rowekamp, W. (1981) *J. Mol. Biol. 153*, 273–289.
Quiocho, F. A. (1986) *Annu. Rev. Biochem. 55*, 287–315.
Ruoho, A. E., Kiefer, H., Roeder, P. E., & Singer, S. J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2567–2571.
Satake, K., Okuyama, T., Ohashi, M., & Shinoda, T. (1960) *J. Biochem. (Tokyo) 47*, 654–659.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–667.
Shanahan, M. F., Wadzinski, B. E., Lowndes, J. M., & Ruoho, A. E. (1985) *J. Biol. Chem. 260*, 10897–10900.
Simpson, D. L., Rosen, S. D., & Barondes, S. H. (1974) *Biochemistry 13*, 3487–3493.
Springer, W. R., Cooper, D. N. W., & Barondes, S. H. (1984) *Cell (Cambridge, Mass.) 39*, 557–564.

CORRECTION

Myosin Isoforms in Normal and Dystrophic Chickens, by Julie Ivory Rushbrook,* Cipora Weiss, Axel Georg Wadewitz, and Alfred Stracher, Volume 26, Number 14, July 14, 1987, pages 4454–4460.

Page 4454. In the published paper, the name of the third author was misspelled. The above version is correct.

Page 4458. In column 2, the first paragraph should read as follows: The fragments of interest were found to differ in sequence at residue 22 (Table I). The first peak of the normal adult 20-kDa triplet contained Ala at this position as did the second peak of the adult dystrophic quadruplet. The first peak of the 5-day posthatch triplet contained Thr at this position, and this residue was also found at position 22 in peak 1′ of the dystrophic chromatogram. The results are consistent with dystrophic myosin containing the isozymes of normal adult and 5-day posthatch myosins.