Modification of Proteoglycans during Maturation of Fibroblast Substratum Adhesion Sites†

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ABSTRACT: Newly formed adhesion sites, left bound to the tissue culture substratum after [ethylenebis(oxyethylenenitrilo)]tetraacetic acid mediated detachment of simian virus 40 transformed Balb/c 3T3 cells, have been extracted with 0.5 M guanidine hydrochloride or Zwittergent (3-12), extractions which identify different subfractions of proteoglycans in these sites. The compositions of these extracts were then compared to similar extracts of "maturing" adhesion sites in an effort to identify structural and metabolic changes which may occur with time and which may play a role in altering adhesion during cell movement. Guanidine hydrochloride (0.5 M) extracts *both* hyaluronate and chondroitin sulfate proteoglycan from newly formed sites (but which are *not* complexed in an aggregate similar to that found in cartilage) but *only* hyaluronate from fully matured sites, indicating that the chondroitin sulfate proteoglycans somehow become resistant to extraction with time. Both high and low molecular weight forms of hyaluronate also accumulate in sites with time. Zwittergent 3-12 solubilizes free chains of heparan sulfate but not heparan sulfate proteoglycan from either class of sites. Most of the heparan sulfate in newly formed sites occurs as a large proteoglycan excludable from Sepharose CL-6B columns under stringent dissociative conditions; however, as adhesion sites "mature", a portion of this proteoglycan appears to be converted by some unknown mechanism to free heparan sulfate chains. This process may very well weaken the close adhesive contacts between the cell and substratum mediated by fibronectin's binding to the highly multivalent heparan sulfate proteoglycans. These studies further indicate that there is considerable metabolism and changing intermolecular associations of proteoglycans within these sites during movement of fibroblasts over this model extracellular matrix.

When fibroblasts growing on a tissue culture substratum are treated with the Ca^{2+}-specific chelating agent [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA),[1] they can be detached from the substratum while leaving focal pools of cellular adhesive material as substratum-attached material (Rosen & Culp, 1977; Culp et al., 1979). This substratum-attached material appears to represent the tight focal contact adhesion sites (Izzard & Lochner, 1976, 1980), as well as some close contact material (Laterra et al., 1983a), through which cells directly interact with the substratum-adsorbed serum proteins. Biochemical characterization of this adhesive material has indicated that these sites are enriched in specific cell surface components (Rollins et al., 1982) which appear to change quantitatively depending on the culture conditions (Rollins & Culp, 1979a,b). When cells are allowed to attach for only a short period of time, their substratum-attached material (referred to as reattaching or R-SAM) is enriched in newly formed footpad adhesion sites (Revel et al., 1974; Rosen & Culp, 1977) which contain an increased amount of heparan sulfate proteoglycan (Rollins & Culp, 1979a,b). With time, cells begin to move in culture and leave their footpad adhesion sites behind as "footprint" material (Rosen & Culp, 1977; Chen, 1977, 1981). This long-term culture-generated substratum-attached material (L-SAM) containing footpad adhesion sites and footprints is comprised of an increased quantity of hyaluronate and chondroitin sulfate proteoglycan (Rollins & Culp, 1979a). It had been proposed that this accumulation of hyaluronic acid leads to reorganization of molecules at the periphery of footpad adhesion sites and then to cytoskeletal disorganization with subsequent detachment of the cell while leaving a footprint remnant bound to the tissue culture substratum (Rollins et al., 1982).

An accumulating body of evidence also indicates that heparan sulfate proteoglycans in substratum-attached material mediate the close contacts of cells by binding to substratum-bound fibronectin (Laterra et al., 1983a). Because of the central role of these proteoglycans and hyaluronate in the adhesive processes of fibroblasts, we have been studying the intermolecular associations of these components both in vitro and in situ (Garner & Culp, 1981; Lark & Culp, 1982; Miketo & Culp, 1983). Two selective extraction conditions have been chosen in an effort to isolate complexes of glycoconjugates which may be interacting within substratum-attached material. A low concentration of guanidine hydrochloride (0.5 M) has previously been shown to selectively solubilize most of the hyaluronic acid while solubilizing very little chondroitin sulfate proteoglycan or heparan sulfate proteoglycan from long-term-generated fibroblast adhesion sites (Lark & Culp, 1982). It is possible that newly formed adhesion sites may contain aggregates of chondroitin sulfate proteoglycans bound to hyaluronate similar to the cartilage system (Faltz et al., 1979) and with time the interaction of these molecules may somehow change. Therefore, this reagent was used to extract newly formed and "maturing" substratum-attached material in an effort to identify organizational changes within these sites which may be occurring with time. Additionally, treatment of long-term-generated adhesion sites with the zwitterionic detergent Zwittergent 3-12 results in extraction of free heparan sulfate chains without solubilization of heparan sulfate pro-

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[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Gdn·HCl, guanidine hydrochloride; L-SAM, long-term metabolically radiolabeled substratum-attached material containing both footpad and footprint adhesive materials as further explained in the text; Na-DodSO$_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; R-SAM, reattaching substratum-attached material enriched in only newly formed footpad adhesion sites; SV40, simian virus 40; EDTA, ethylenediaminetetraacetic acid; TRU, turbidity reducing unit.

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teoglycans (Lark & Culp, 1982). Since newly formed adhesion sites are highly enriched in heparan sulfate, this reagent was used to determine the possible conversion of heparan sulfate proteoglycans to free heparan sulfate chains which may be occurring with maturation of the adhesion site. This study describes a number of changes which occur in the organization of various components within the adhesion site including heparan sulfate proteoglycan, chondroitin sulfate proteoglycan, and hyaluronic acid. These changes are discussed relative to the presumed roles that these molecules may play in the adherence and subsequent detachment of a cell to this model extracellular matrix.

Experimental Procedures

Materials were supplied as follows: D-[6-^{3}H]glucosamine hydrochloride, L-[4,5-^{3}H]leucine, and $Na_2{}^{35}SO_4$ from Amersham Corp.; NEF-963 aqueous scintillation cocktail from New England Nuclear Corp.; Pronase and Zwittergent 3-12 from Calbiochem-Boehring Corp.; *Streptomyces* hyaluronidase from Miles Laboratories, Inc.; testicular hyaluronidase from Worthington Biochemical Corp.; guanidine hydrochloride from Bethesda Research Laboratories; sodium dodecyl sulfate from Bio-Rad Laboratories; Sepharose CL-2B, Sepharose CL-6B, and Sephacryl S-200 Superfine from Pharmacia Fine Chemicals, Inc.; EGTA from Eastman Organic Chemicals; Eagle's minimal essential medium from Grand Island Biologicals Co.; donor calf serum from K. C. Biologicals, Inc.; plastic tissue culture dishes from Lux Scientific Co.; CX-10 untrafiltration units from Millipore Corp.

Cell Culture. Mycoplasma-free SV40 transformed Balb/c 3T3 cells (clone SVT2) were cultured between their 15th and 25th passages in Eagle's minimal essential medium supplemented with 4 times the essential amino acids and vitamins, 10% donor calf serum, 250 units/mL penicillin, and 0.25 mg/mL streptomycin sulfate at 37 °C in humidified 5% CO_2–95% air.

Radiolabeling and Isolation of Substratum-Attached Material. Long-term-radiolabeled substratum adhesion sites, containing both footpad and footprint adhesive material, were isolated as previously described and abbreviated as L-SAM[1] (Garner & Culp, 1981). Briefly, 7.5×10^5 cells were inoculated into each of 64 or 96 100-mm diameter tissue culture dishes containing 7 mL of complete medium. After 3 h in culture to allow for stable attachment and metabolic recovery, the medium was removed and replaced with 7 mL of one of the following: complete medium containing 5 μCi/mL [6-^{3}H]glucosamine, 90% leucine-depleted medium containing 5 μCi/mL [4,5-^{3}H]leucine, or streptomycin sulfate free medium containing 50 μCi/mL $Na_2{}^{35}SO_4$. After incubation for 72 h of exponential growth in the radiolabeling medium, the cells were rinsed twice with PBS and detached from the tissue culture substratum by shaking the dishes in 5 mL of 0.5 mM EGTA in PBS at 37 °C for 30 min, followed by gentle pipetting. The L-SAM remaining on the tissue culture substratum was extracted with (a) 0.2% (w/v) $NaDodSO_4$ in buffer I (buffer I: 150 mM sodium acetate, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 5.8) at 37 °C for 30 min, (b) 0.2% Zwittergent 3-12 (w/v) in buffer II containing protease inhibitors (buffer II: 50 mM sodium acetate, 50 mM benzamidine hydrochloride, 100 mM 6-aminohexanoic acid, and 10 mM EDTA, pH 5.8) at 4 °C for 30 min, or (c) 0.5 M guanidine hydrochloride (Gdn·HCl)[1] in buffer II at 4 °C for 4 h. These conditions allow for maximal extraction of substratum-attached material ($NaDodSO_4$ solubilizes all of the radiolabeled material whereas the other two reagents solubilize only a subset of the radiolabeled components).

To isolate newly formed adhesion sites, that is reattaching substratum-attached material (R-SAM)[1] (Rollins et al., 1982), approximately 8×10^8 long-term-radiolabeled cells pooled after EGTA-mediated detachment were inoculated into each of 32 or 48 100-mm tissue culture dishes containing complete medium without any radioisotope. After 1 h in culture to allow formation of new footpad adhesion sites (Rosen & Culp, 1977), the medium was removed, the cells were detached with EGTA, and R-SAM was isolated as described for L-SAM. R-SAM was then extracted with 0.2% $NaDodSO_4$ in buffer I, 0.2% Zwittergent 3-12 in buffer II, or 0.5 M Gdn·HCl in buffer II under the same extraction conditions as described previously for L-SAM (Lark & Culp, 1982). The Gdn·HCl and Zwittergent extracts were concentrated at 4 °C by vacuum dialysis; the $NaDodSO_4$ extracts were concentrated by using Millipore CX-10 filters at room temperature.

Gel Filtration Chromatography. Both R-SAM and L-SAM extracts were chromatographed on identical Sepharose CL-2B (0.8 × 60 cm), Sepharose CL-6B (1 × 120 cm), or Sephacryl S-200 superfine (1 × 120 cm) columns. Samples were eluted with the same buffer used for extraction at a flow rate of 6–10 mL/h, and an aliquot of each fraction was assayed for radioactivity by scintillation counting. Due to nonspecific adsorption of some of the Gdn·HCl- and Zwittergent-extracted material to the column matrix, yields were initially as low as 50%, but with continued use of the same column recoveries increased to 75–85%. Profiles from control and treated samples were normalized so that direct comparisons could be made.

Quantitation of Solubilized Substratum-Attached Material. Both long-term and newly formed substratum adhesion sites were extracted with Gdn·HCl or Zwittergent as described above. The tissue culture dishes from which the adhesive material was extracted were subsequently soaked overnight at 4 °C in PBS to dissociate Gdn·HCl bound to proteins (Gdn·HCl forms precipitates when mixed with solutions of $NaDodSO_4$). The plates were then rinsed twice in PBS and twice in distilled water prior to incubation at 37 °C for 30 min in 5 mL of 0.2% $NaDodSO_4$, which quantitatively solubilizes all of the radiolabeled substratum-attached material (Cathcart & Culp, 1979). The quantity of material solubilized with each extractant was determined by dividing the amount of material solubilized with Gdn·HCl or Zwittergent alone by the amount of material removed with the solvent plus the amount subsequently removed with $NaDodSO_4$.

Enzyme Digestion. Prior to enzymatic digestion, all Gdn·HCl and Zwittergent solubilized material was dialyzed overnight at 4 °C against the appropriate buffer: (a) for *Streptomyces* hyaluronidase digestion (4 h at 37 °C with 10 TRU/mL enzyme) against buffer III (buffer III: 100 mM sodium acetate, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 5.8) and (b) for testicular hyaluronidase digestion (4 h at 37 °C with 10 μg/mL enzyme) against buffer I. Adhesive material resistant to either Gdn·HCl or Zwittergent solubilization was subsequently extracted with $NaDodSO_4$ and dialyzed for 48 h at room temperature against the enzymatic digestion buffer discussed above prior to treatment with the enzymes (under conditions in which any residual $NaDodSO_4$ was not inhibitory to the particular enzyme being used). After digestion, all samples were brought to a final concentration of 0.2% $NaDodSO_4$ and chromatographed on a Sepharose CL-6B column eluted with 0.2% $NaDodSO_4$ in buffer I.

Alkaline–Borohydride Reduction. Samples were treated with alkali and sodium borohydride under conditions which cleave protein–polysaccharide linkages (Carlson, 1968; Ogata & Lloyd, 1982) but which prevent "peeling" of long poly-

Table I: Solubility of Glycoconjugates from Adhesion Sites with Gdn·HCl or Zwittergent

type of SAM	extractant	radioactivity (percentage of total) [^{3}H]-glucosamine	[^{3}H]-leucine	$Na_2{}^{35}SO_4$
L-SAM[a]	Gdn·HCl	30 ± 4	53 ± 9	26 ± 8
	Zwittergent	65 ± 6	73 ± 6	57 ± 12
R-SAM[b]	Gdn·HCl	26 ± 3	32 ± 14	22 ± 8
	Zwittergent	79 ± 6	24 ± 4	61 ± 10

[a] Long-term metabolically radiolabeled substratum-attached material containing footpad and footprint adhesive material was isolated and extracted with either Gdn·HCl or Zwittergent. The material resistant to these treatments was then solubilized with $NaDodSO_4$. The percentage of extractable material is expressed as the percentage of total radiolabeled L-SAM extractable with $NaDodSO_4$ (Cathcart & Culp, 1978). [b] Reattaching substratum-attached material containing principally footpad adhesive material was isolated from metabolically radiolabeled cells (as indicated) after 1 h of attachment. The isolated material was then extracted with either Gdn·HCl or Zwittergent, followed by solubilization of resistant material with $NaDodSO_4$, as discussed under Experimental Procedures. The percentage of extractable material is expressed as the percentage of total radiolabeled R-SAM extractable with $NaDodSO_4$.

saccharide chains (Spiro, 1972). Samples were dialyzed overnight against buffer I and split into control and experimental halves. To the experimental half was added an equal volume of 7.5% sodium borohydride in 0.1 M sodium hydroxide to a final concentration of 1.0 M sodium borohydride in 0.05 M sodium hydroxide for incubation at 45 °C for 19 h. Samples were neutralized with glacial acetic acid, brought to 0.2% $NaDodSO_4$, and chromatographed on Sepharose CL-6B or Sephacryl S-200 superfine columns eluted with 0.2% $NaDodSO_4$ in buffer I.

Nitrous Acid Deamination. All samples were dialyzed overnight at room temperature against glass-distilled water prior to treatment with nitrous acid to test for heparan sulfate content (Lindahl et al., 1973). Sodium nitrite in 1.8 M acetic acid was added to experimental samples to a final concentration of 1.8% while acetic acid alone was added to control samples. All samples were incubated for 80 min at room temperature, and the reaction was terminated by addition of an equal volume of 2 M ammonium sulfamate. All samples were brought to 0.2% in $NaDodSO_4$ and chromatographed on identical Sephacryl S-200 superfine columns eluted with 0.2% $NaDodSO_4$ in buffer I.

Results

Solubility of Adhesion Site Macromolecules with Gdn·HCl or Zwittergent. Neither Gdn·HCl nor Zwittergent alone solubilizes all of the [^{3}H]leucine-, [^{3}H]glucosamine-, or $^{35}SO_4{}^{2-}$-radiolabeled R-SAM (Table I). Zwittergent extracts about three-fourths of the [^{3}H]glucosamine-radiolabeled glycoconjugate while extracting slightly over half of the $^{35}SO_4{}^{2-}$-radiolabeled GAG and about one-fourth of the protein. By comparison, approximately three-fourths of the protein, two-thirds of the $^{35}SO_4{}^{2-}$-radiolabeled GAG, and two-thirds of the amino sugar radiolabeled material from L-SAM is extracted under these same conditions with Zwittergent (Lark & Culp, 1982) (Table I). On the other hand, 0.5 M Gdn·HCl extracts less than one-third of the protein, glucosamine-radiolabeled material, and $^{35}SO_4{}^{2-}$–GAG from R-SAM while solubilizing similar amounts of $^{35}SO_4{}^{2-}$–GAG and glucosamine-radiolabeled glycoconjugate but twice as much protein from L-SAM (Table I). When Gdn·HCl and Zwittergent are used together to extract either L-SAM or R-SAM, over 90% of the radiolabeled material can be solubilized (data not shown). Any number of changes may be occurring within the adhesion site with time, which alter the sensitivity of these radiolabeled pools of material to extraction with Gdn·HCl or Zwittergent.

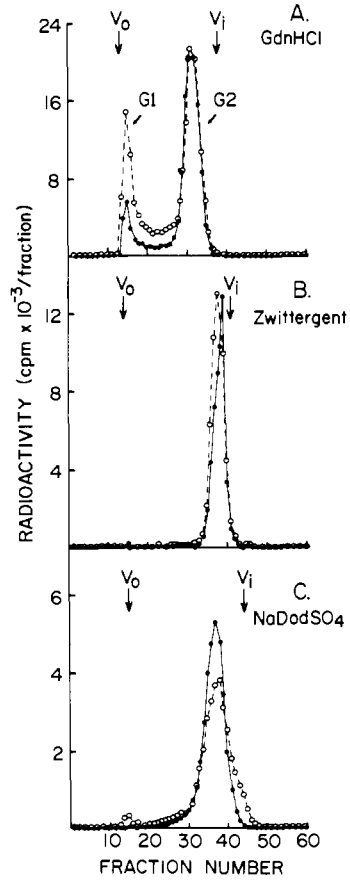

FIGURE 1: Gel filtration chromatography of R-SAM and L-SAM solubilized with various reagents. SVT2 cells were metabolically radiolabeled with [^{3}H]glucosamine for 72 h, after which time both R-SAM and L-SAM were isolated as described under Experimental Procedures. The isolated substratum-attached material was then extracted with 0.5 M Gdn·HCl in buffer II, 0.2% Zwittergent in buffer II, or 0.2% $NaDodSO_4$ in buffer I. Solubilized L-SAM (○) or R-SAM (●) was concentrated and chromatographed over Sepharose CL-2B columns (0.8 × 60 cm) eluted with the same buffer used for extraction: (A) 0.5 M Gdn·HCl in buffer II; (B) 0.2% Zwittergent in buffer II; (C) 0.2% $NaDodSO_4$ in buffer I. The V_0 and V_i regions were identified with blue dextran and dinitrophenylated glycine, respectively.

Gel Filtration of Molecules Solubilized with Gdn·HCl or Zwittergent. In order to initially characterize the glycoconjugates solubilized with the two reagents, [^{3}H]glucosamine-radiolabeled L-SAM or R-SAM was extracted and the solubilized material chromatographed over identical Sepharose CL-2B columns (Figure 1). Although Gdn·HCl solubilizes similar quantities of glucosamine-radiolabeled glycoconjugate from both L-SAM and R-SAM (Table I), the Gdn·HCl-soluble material from R-SAM (Figure 1A) contains about half as much material eluting in the V_0 region of the profile (G1) when compared to the Gdn·HCl extract of L-SAM. The Gdn·HCl-soluble material (Figure 1A) is highly enriched in G1 in comparison to the $NaDodSO_4$ extracts of either L-SAM or R-SAM (Figure 1C). The R-SAM G1 material could be either hyaluronate similar to that identified in the extracts of L-SAM (Lark & Culp, 1982) or a large multimolecular aggregate (see below).

[^{3}H]Glucosamine-radiolabeled L-SAM or R-SAM sensitive to Zwittergent extraction elutes almost identically from a Sepharose CL-2B column (Figure 1B). These extracts are

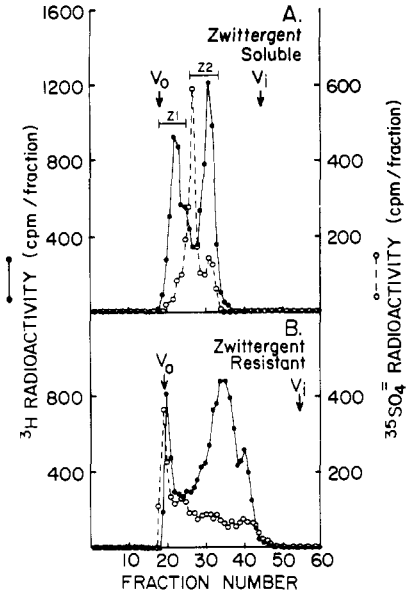

FIGURE 2: Gel filtration chromatography of Zwittergent-soluble and -resistant materials. R-SAM was extracted with 0.2% Zwittergent, followed by solubilization of the Zwittergent-resistant material with $NaDodSO_4$ (see Experimental Procedures). (A) Both [^{3}H]glucosamine (●) and $^{35}SO_4^{2-}$- (○) radiolabeled R-SAM solubilized with Zwittergent was concentrated and chromatographed over a Sephacryl S-200 column (1 × 120 cm) equilibrated with 0.2% Zwittergent in buffer II to permit direct size comparisons. (B) Both the [^{3}H]-glucosamine- (●) and $^{35}SO_4^{2-}$- (○) radiolabeled portions of R-SAM resistant to Zwittergent extraction were solubilized with $NaDodSO_4$, and the concentrated extracts were chromatographed over the same Sepharose CL-6B column (1 × 100 cm) eluted with 0.2% $NaDodSO_4$ in buffer I so that the elution profiles could be compared. The V_0 and V_i regions of the Sephacryl S-200 column were identified with high molecular weight [^{3}H]hyaluronic acid and [^{3}H]glucosamine, respectively. The V_0 and V_i regions of the Sepharose CL-6B column were identified with blue dextran and dinitrophenylated glycine, respectively.

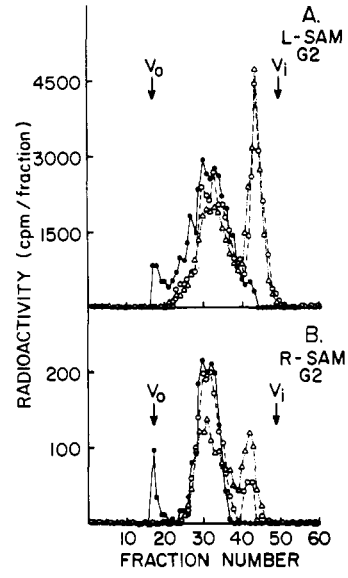

FIGURE 3: Digestion of Gdn·HCl-extracted glycoconjugate with hyaluronidase. [^{3}H]Glucosamine-radiolabeled G2 material (Figure 1A) was isolated and concentrated prior to enzymatic treatment as discussed under Experimental Procedures. (A) G2 material from Gdn·HCl-extracted L-SAM was split into three portions, and each third was treated with *Streptomyces* hyaluronidase (○) and testicular hyaluronidase (△) or mock digested (●). (B) G2, isolated from R-SAM, was also digested with *Streptomyces* hyaluronidase (○) and testicular hyaluronidase (△) or treated as a control (●). After enzymatic digestion, each sample was brought to a final concentration of 0.2% in $NaDodSO_4$ prior to chromatography over the same Sepharose CL-6B column (1 × 120 cm) equilibrated with 0.2% $NaDodSO_4$ in buffer I. The V_0 and V_i regions were identified with blue dextran and dinitrophenylated glycine, respectively.

enriched in glycoconjugates smaller than those found in Gdn·HCl-soluble material as demonstrated by their proximity to the V_i region of the profile. In order to further fractionate the R-SAM solubilized with Zwittergent, this material was chromatographed over a Sephacryl S-200 column (Figure 2A). The [^{3}H]glucosamine-radiolabeled material eluted as two peaks of radioactivity (Z1 and Z2), each accounting for about half of the radioactivity recovered from the column. The majority of the $^{35}SO_4^{2-}$-radiolabeled R-SAM solubilized with Zwittergent (Figure 2A) elutes between Z1 and Z2; however, a small amount of radioactivity is noted in the Z2 region of the profile. There is an increased quantity of Z1 in the Zwittergent-solubilized R-SAM relative to that solubilized from L-SAM (Lark & Culp, 1982).

Both the [^{3}H]glucosamine- and $^{35}SO_4^{2-}$-radiolabeled portions of R-SAM resistant to Zwittergent extraction but subsequently extracted with Na_2DodSO_4 (Figure 2B) are enriched in material eluting in the V_0 region of a Sepharose CL-6B column. This indicates that most of the high molecular weight glycoconjugates resist Zwittergent extraction. Also, comparison of the Sepharose CL-6B gel filtration profiles of amino sugar radiolabeled L-SAM and R-SAM resistant to Zwittergent extraction indicates that the L-SAM generates much more material eluting in the V_0 region of the profile than the resistant material isolated from R-SAM. This correlates with the fact that R-SAM contains much less hyaluronic acid than L-SAM (Rollins & Culp, 1979a), and this V_0 material isolated from the L-SAM extract has been identified as hyaluronate (Lark & Culp, 1982).

Hyaluronate and Chondroitin Sulfate Contents of the Gdn·HCl Extracts. The [^{3}H]glucosamine-radiolabeled L-SAM material solubilized with $NaDodSO_4$ which elutes in the V_0 region of a Sepharose CL-2B column has previously been identified as only hyaluronic acid (Rollins & Culp, 1979a,b), as has the G1 pool of material isolated from a Gdn·HCl extract of L-SAM (Figure 1A) [see Lark & Culp (1982)]. Therefore, G1 material isolated from R-SAM (Figure 1A) was treated with *Streptomyces* hyaluronidase, which is specific for digesting hyaluronic acid, and has been shown to be completely sensitive (data not shown), indicating that it is entirely hyaluronate. $NaDodSO_4$ treatment does not cause a shift of any of the G1 material from the V_0 region to a more included position on Sepharose CL-2B or CL-6B columns (data not shown), suggesting that G1 is not an aggregate of molecules. When $^{35}SO_4^{2-}$-radiolabeled R-SAM was extracted with Gdn·HCl and this extract chromatographed over a Sepharose CL-2B column, none of the radiolabeled material eluted in the G1 area of the profile (data not shown), further evidence that multimolecular aggregates containing sulfated GAG's are not present in this extract.

To characterize hyaluronate and chondroitin sulfate content of the G2 portion (Figure 1A) of the Gdn·HCl extract, G2 was isolated and treated with *Streptomyces* hyaluronidase or testicular hyaluronidase (Figure 3) as described under Experimental Procedures. Slightly less than half of the G2 material isolated from L-SAM shifts toward the V_i region of a Sepharose CL-6B column when treated with the *Streptomyces* enzyme (Figure 3A), indicating a sizable amount of hyaluronate. However, when the same material is digested with testicular hyaluronidase, there is only a slight increase in the shift of material into V_i relative to the *Streptomyces* hyaluronidase-induced shift, indicating that there is very little

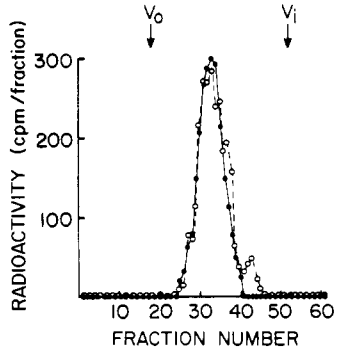

FIGURE 4: *Streptomyces* hyaluronidase digestion of Z1. [^{3}H]-Glucosamine-radiolabeled Z1 (see Figure 2) was isolated, concentrated, and split in half prior to enzymatic digestion (see Experimental Procedures). Half of the extract was digested with *Streptomyces* hyaluronidase (O) while the other half was handled as a control (●). All samples were brought to 0.2% in $NaDodSO_4$ prior to chromatography over a Sepharose CL-6B column (1 × 120 cm) equilibrated with 0.2% $NaDodSO_4$ in buffer I. The V_0 and V_i regions were defined as described in Figure 3.

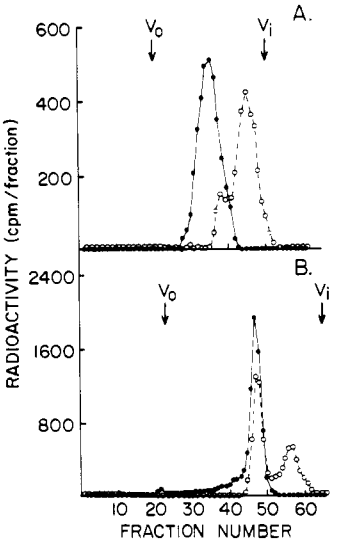

FIGURE 5: Alkaline–borohydride reduction of Zwittergent-solubilized glycoconjugates. [^{3}H]Glucosamine-radiolabeled R-SAM extracted with Zwittergent was separated into two peaks of material identified as Z1 and Z2 (see Figure 2). Both Z1 and Z2 were pooled, concentrated, and split in half prior to alkaline–borohydride reduction (see Experimental Procedures). (A) Half of Z1 was treated with alkaline–sodium borohydride (O) while the other half was handled as a control (●). (B) Half of Z2 was also reduced with alkaline–borohydride (O) while the other half was treated as a control (●). All samples were brought to neutral pH prior to chromatography over a Sepharose CL-6B column (1 × 120 cm) equilibrated with 0.2% $NaDodSO_4$ in buffer I. The V_0 and V_i regions of the profiles were identified as described in Figure 3.

chondroitin sulfate in this extract (chondroitin sulfate being sensitive to the testicular enzyme but not to the *Streptomyces* enzyme, whereas hyaluronate is equally sensitive to either enzyme). Analysis of the Gdn·HCl-resistant L-SAM with these enzymes revealed that most of the chondroitin sulfate in L-SAM could be recovered in this fraction (Lark & Culp, 1982).

G2 material from R-SAM was treated similarly with the two hyaluronidases. About 10% of the G2 material is sensitive to *Streptomyces* hyaluronidase (Figure 3B), revealing that there is only a *small* amount of hyaluronic acid in the G2 material from R-SAM. When the same material is treated with testicular hyaluronidase (Figure 3B), approximately one-third of the extract shows sensitivity to the enzyme. It therefore appears that hyaluronate and chondroitin sulfate moieties are cosolubilized from R-SAM with 0.5 M Gdn·HCl in comparison to the selective solubilization of hyaluronate alone from L-SAM under these same conditions.

Hyaluronic Acid Content of Zwittergent Extract. To assay for hyaluronate in the Zwittergent-soluble R-SAM, Z1 and Z2 (see Figure 2A) were pooled separately and treated with *Streptomyces* hyaluronidase. Upon digestion of Z1 with the enzyme (Figure 4), a small amount of material shifts to a further included region of a Sepharose CL-6B column reflecting a small amount of hyaluronic acid. Z2 was similarly treated (data not shown); however, none of the material was sensitive to the enzyme. *Streptomyces* hyaluronidase digestion of the Zwittergent resistant R-SAM (see Figure 2B) results in a shift of part of the material eluting in the V_0 region of a Sepharose CL-6B profile (data not shown), indicating that the high molecular weight class of hyaluronate resists Zwittergent extraction. These results indicate that Zwittergent solubilizes only a portion of the hyaluronate and that which is solubilized is relatively small as indicated by its elution within Z1 from a Sephacryl S-200 column.

Glycoprotein Content of Zwittergent-Soluble Material. Since Zwittergent-solubilized L-SAM material is enriched in glycoprotein (Lark & Culp, 1982), Zwittergent-solubilized R-SAM was also analyzed for this class of glycoconjugate. To identify covalent protein–polysaccharide linkages, the material was treated with alkaline–sodium borohydride under conditions which lead to breakdown of protein–polysaccharide linkages (Carlson, 1968; Ogata & Lloyd, 1982). After such a treatment of amino sugar radiolabeled Z1 (Figure 5A), most of the material shifts to a more included position on a Sepharose CL-6B column, indicating that virtually all of this polysaccharide is linked to protein. $^{35}SO_4^{2-}$-radiolabeled R-SAM extracted with Zwittergent elutes from a Sephacryl S-200 column (see Figure 2A) as two peaks of radioactivity, the first between Z1 and Z2 and the second in the Z2 area of the profile, suggesting that Z1 contains very little sulfated material. This low degree of sulfation, the homogeneity of the reduction products, the insensitivity of most of the material to glycosaminoglycan lyases, and the low molecular weight of Z1 suggest that the majority of this material is glycoprotein.

When Z2 from R-SAM is treated with alkaline–borohydride under the same conditions as discussed for Z1 (Figure 5B), less than half of the material shifts to a further included position on a Sepharose CL-6B column, indicating that only a portion of Z2 is protein covalently attached to polysaccharide. Since Z2 contains some sulfated polysaccharide, the amino sugar radiolabeled material sensitive to alkaline–borohydride could be a sulfated proteoglycan and/or glycoprotein. Upon treatment of amino sugar radiolabeled Z2 with *Streptomyces* hyaluronidase, testicular hyaluronidase, or nitrous acid to identify hyaluronate, chondroitin sulfate, or highly N-sulfated heparan sulfate, respectively (data not shown), none of this material shifts to a further included position on a Sepharose CL-6B column, indicating that Z2 probably contains very little of these components even though a small amount of GAG can be detected by sulfate radiolabeling. The relative lack of these glycosaminoglycans and the similarity in elution profiles of the reduction products of Z1 and Z2 suggest that most of the Z2 material sensitive to alkaline–borohydride is also glycoprotein. When similar analytical approaches are used, a portion of Z2 isolated from L-SAM is also found to resist alkaline–borohydride reduction. In contrast to the Zwittergent soluble R-SAM analyses described above, characterization of L-SAM material indicates that it is enriched in free, highly N-sulfated chains of heparan sulfate not linked to protein

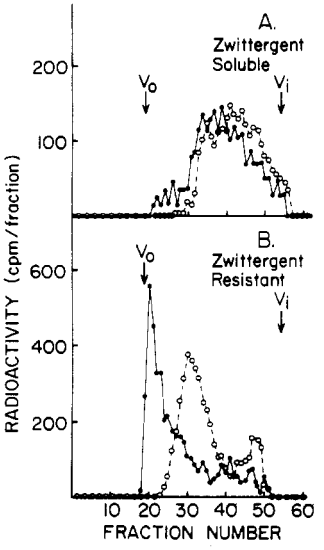

FIGURE 6: Sensitivity of sulfate-radiolabeled R-SAM to alkaline–borohydride reduction. $^{35}SO_4^{2-}$-radiolabeled R-SAM was first extracted with Zwittergent, followed by solubilization of the Zwittergent-resistant material with $NaDodSO_4$. Both the Zwittergent-soluble and -resistant materials were concentrated and split in half prior to alkaline–borohydride reduction. (A) Half of the Zwittergent-soluble material was treated with alkaline–borohydride (○) while the other half was handled as a control (●). (B) Each half of the Zwittergent-resistant material solubilized with $NaDodSO_4$ was also either treated with alkaline–borohydride (○) or treated as a control (●). All samples were brought to neutral pH prior to chromatography over a Sepharose CL-6B column (1 × 120 cm) eluted with 0.2% $NaDodSO_4$ in buffer I. Both the V_0 and V_i regions were determined as in Figure 3.

(Lark & Culp, 1982). A similar analysis was performed on the *amino sugar radiolabeled* Z2 material from R-SAM which resists alkaline–borohydride reduction. Nitrous acid deamination of this material results in a shift of essentially *none of the material* on a Sepharose CL-6B column, indicating that there is no free chain heparan sulfate in this portion of the R-SAM extract.

Sulfated Proteoglycan Content of Zwittergent-Soluble Material. Most of the $^{35}SO_4^{2-}$-radiolabeled material solubilized with Zwittergent elutes between Z1 and Z2 (Figure 2A). Therefore, $^{35}SO_4^{2-}$-radiolabeled R-SAM was extracted and analyzed for sulfated glycosaminoglycan and proteoglycan content. Alkaline–borohydride reduction of this material (Figure 6A) results in a shift of only a small portion of the ^{35}S radioactivity into a further included region of the Sepharose CL-6B column profile. This suggests that most of the $^{35}SO_4^{2-}$-radiolabeled R-SAM extracted with Zwittergent is probably free chains of glycosaminoglycans and not proteoglycan. On the other hand, alkaline–borohydride reduction of the Zwittergent-resistant portion of R-SAM (Figure 6B) results in a shift of all of the radiolabeled material to a more included position on a Sepharose CL-6B column, indicating this $^{35}SO_4^{2-}$-radiolabeled material is proteoglycan.

To quantitate the highly N-sulfated heparan sulfate content of both the Zwittergent-soluble and resistant pools of R-SAM, both of these extracts were treated with nitrous acid. This treatment of the ^{35}S-radiolabeled and solubilized pool (Figure 7A) generates a shift of approximately one-third of the material in the Sepharose profile. The material resistant to deamination probably contains both undersulfated heparan sulfate and some chondroitin sulfate proteoglycan.

Upon nitrous acid deamination of the pool of $^{35}SO_4^{2-}$-radiolabeled R-SAM resistant to Zwittergent extraction (Figure 7B) to identify N-sulfated heparan sulfate, more than two-thirds of the material shifts to a more included position

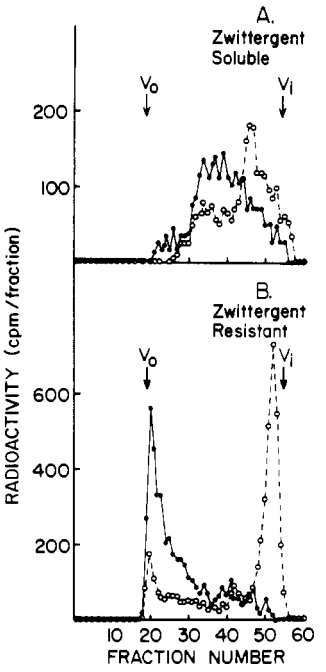

FIGURE 7: Nitrous acid deamination of sulfate-radiolabeled glycoconjugates. $^{35}SO_4^{2-}$-radiolabeled R-SAM's solubilized with Zwittergent or subsequently with $NaDodSO_4$ (Zwittergent-resistant material) were pooled, concentrated, and split in half prior to nitrous acid deamination as described under Experimental Procedures. (A) Half of the Zwittergent-soluble material was treated with nitrous acid (○) while the other half was treated with only acetic acid as a control (●). (B) One portion of the Zwittergent-resistant material extracted with $NaDodSO_4$ was treated with nitrous acid (○) while the other half was handled as a control (●). All samples were brought to 0.2% in $NaDodSO_4$ followed by chromatography over a Sepharose CL-6B column (1 × 120 cm) equilibrated with 0.2% $NaDodSO_4$. Both V_0 and V_i were identified as in Figure 3.

on a Sepharose CL-6B column. This heparan sulfate proteoglycan is sufficiently large to be excluded from the Sepharose CL-6B column, and it elutes with a K_{av} of 0.6 from a Sepharose CL-2B column during elution with $NaDodSO_4$-containing buffer (Figure 7B) and is entirely proteoglycan as indicated by its complete sensitivity to alkaline–borohydride reduction (see above).

Discussion

Fibroblasts attach to a serum-adsorbed tissue cultue substratum through focal patches of the cell undersurface visualized as footpad adhesion sites in the scanning electron microscope (Revel et al., 1974; Rosen & Culp, 1977). These adhesion sites have been isolated as substratum-attached material (Culp et al., 1979), which contains both tight focal contact material and close contact adhesive material (Izzard & Lochner, 1976, 1980; Laterra et al., 1983a,b) and which appears to vary in biochemical composition depending on the length of time the cells are in contact with the substratum (Culp et al., 1979). When cells initially attach and spread, their adhesion sites are enriched in heparan sulfate containing entities, but with time there is an accumulation of hyaluronate and chondroitin sulfate. Izzard & Lochner (1980) have shown that fibroblasts initially attach to a fibronectin-containing substratum through close contact adhesion sites of about 30-nm spacing distance between the cell's undersurface and the substratum; however, with time tight focal contacts of about 10–15-nm distance appear within these close contacts. Proteoglycans isolated from both newly formed and more mature adhesion sites have the capacity to aggregate into multimolecular complexes (Garner & Culp, 1981; Miketo & Culp,

1983); however, these aggregates are compositionally quite different. Therefore, it appears that a number of both compositional and organizational changes occur within these substratum adhesion sites from the time of initial attachment through maturation of this adhesive material during which time the cell moves along the substratum and leaves fully matured sites as footprints.

Since adhesion sites are enriched in glycosaminoglycans and proteoglycans which play important roles in the adhesive process (Rollins et al., 1982; Laterra et al., 1980, 1983b), both newly formed and matured substratum-attached material was extracted with solubilization conditions which select for certain subsets of these components (Lark & Culp, 1982). We have used this selectivity to identify structural and metabolic changes in these components which occur with time and which may affect cell adhesion and movement. Low concentrations of Gdn·HCl cosolubilize both hyaluronic acid and some chondroitin sulfate proteoglycans from R-SAM; however, the same treatment results in extraction of only hyaluronate from L-SAM without solubilizing any of the chondroitin sulfate which occurs in sizable concentrations in L-SAM. Zwittergent also appears to extract a small amount of both hyaluronate and chondroitin sulfate proteoglycan from R-SAM, but these components appear to have a lower molecular weight relative to that identified in the Gdn·HCl extract. Since the strong ionic detergent $NaDodSO_4$ appears to have very little effect on the elution of the hyaluronate and chondroitin sulfate isolated from R-SAM with Gdn·HCl upon Sepharose chromatography, these components are probably not complexed in an aggregate similar to that identified from the rat chondrosarcoma tumor (Faltz et al., 1979) or the human diploid fibroblast system (Coster et al., 1979).

It appears then that both hyaluronate and chondrotin sulfate proteoglycan are initially associated with other components in the newly formed adhesion site through a Gdn·HCl-sensitive mechanism but with time there is a change in the chondroitin sulfate proteoglycan resulting in its resistance to Gdn·HCl solubilization. Testicular hyaluronidase digestion in situ of substratum-attached material (Culp et al., 1978) results in solubilization of both hyaluronic acid and chondroitin sulfate, along with all of the newly synthesized and metabolically labile fibronectin. It has also recently been shown that hyaluronate and heparan sulfate have the capacity to interact with multivalent aggregated cell surface fibronectin in vitro (Yamada et al., 1980; Laterra & Culp, 1982). Taken together, these results suggest that within the adhesion site, hyaluronate has the capacity to bind to aggregated cell surface fibronectin, which is also enriched in substratum-attached material (Culp, 1976). However, more direct studies relating to this possibility must be addressed.

This study also indicates that there is both low and high molecular weight size classes of hyaluronate and with time both of these forms of the molecule accumulate within the adhesion site. The increase in hyaluronic acid content with time agrees with results previously reported by Rollins & Culp (1979a,b). It has been proposed that the increase in hyaluronate in more mature or "aged" sites may disrupt the interaction between heparan sulfate proteoglycan and cell surface fibronectin by competing for binding to aggregated cell surface fibronectin (Rollins et al., 1982; Laterra & Culp, 1982). The change in the heparan sulfate proteoglycan–fibronectin interaction would in turn lead to cytoskeletal disorganization and eventual detachment of the cell body from the footpad adhesion site leaving a footprint remnant attached to the substratum. Both of these size classes of hyaluronate appear to increase proportionally with time. The interaction between cell surface fibronectin and hyaluronic acid in vitro is not dependent on the size of the glycosaminoglycan chain (Laterra & Culp, 1982); therefore, one or both of these classes of molecules may play a role in cellular detachment.

An accumulating body of evidence indicates that localized concentrations of multivalent heparan sulfate proteoglycans play a central role in forming adhesions to the substratum (Culp et al., 1979; Rollins et al., 1982; Laterra et al., 1983b), particularly with close contact-type adhesions (Laterra et al., 1983a), by interacting with extracellular glycoproteins such as cell surface and plasma-derived fibronectin. There is an enrichment for heparan sulfate in newly formed adhesion sites (Rollins & Culp, 1979a,b) relative to more mature sites containing both footpad and footprint adhesive material, and close contacts are formed initially by the adhering fibroblast (Izzard & Lochner, 1976, 1980). Since heparan sulfate is found as both proteoglycan and free chains within long-term-generated substratum adhesion sites (Lark & Culp, 1982), and since sulfated glycosaminoglycans are known to undergo differential degradation depending on various culture conditions (David & Bernfield, 1979, 1981), Zwittergent was used to identify the amount of proteoglycan in the adhesive material as adhesion sites are formed and mature during cell movement. Analysis of the Zwittergent extracts indicates that the majority of heparan sulfate in newly formed adhesion sites is proteoglycan, resistant to Zwittergent extraction. Comparatively, over two-thirds of the heparan sulfate in mature adhesion sites is found as free glycosaminoglycan chains which are solubilized with Zwittergent. These data suggest that some of the heparan sulfate proteoglycan is catabolized into smaller molecules by one of two possible mechanisms: the large heparan sulfate proteoglycan identified in R-SAM is first broken down to very small proteoglycans by proteolysis and ultimately to glycopeptides with only a few amino acids covalently linked to the chains, or alternatively, the large heparan sulfate proteoglycan is catabolized by endoglycosidases to liberate free heparan sulfate chains directly as discussed by Robinson et al. (1978) for rat tail heparin proteoglycans. The experiments reported here cannot resolve these two possibilities. Also, R-SAM has been shown to contain a high density heparan sulfate proteoglycan which persists at the bottom of dissociative isopycnic CsCl density gradients while most of the heparan sulfate isolated from L-SAM appears in the low density fraction under identical conditions (Garner & Culp, 1981; Miketo & Culp, 1983). The differential solubilization of free chain heparan sulfate and heparan sulfate proteoglycan with Zwittergent suggests that these two classes of heparan sulfate may be interacting with other cell surface components through different mechanisms. It is possible that free chain heparan sulfate interacts with a membrane "receptor", while the proteoglycan interacts directly with the plasma membrane as described by Kjellen et al. (1981) for hepatocyte proteoglycan which contains a hydrophobic amino acid sequence. Since heparan sulfate appears to play a central role in the organization of close adhesive contacts, it is possible that the conversion from heparan sulfate proteoglycan into free glycosaminoglycan chains may be a reflection of the formation of tight focal contacts within close adhesive contacts and the gradual disappaerance of the close adhesive contacts at the trailing edge of the moving cell. Since L-SAM contains both footpads and footprints while R-SAM is enriched in only footpad adhesion sites, it is also possible that the free chain heparan sulfate will be found only in the footprint material after the cell body has separated from its adhesion sites.

In any case, these experiments have demonstrated very significant metabolic activity in the substratum adhesion sites of these fibroblastic cells, particularly with regard to the conversion of heparan sulfate proteoglycan to free chains. These chains would only be univalent or at most bivalent for binding the adhesive glycoprotein fibronectin, and valency of binding is a most important determinant of adhesive bond formation (Rollins et al., 1982). It would therefore be expected that catabolism of this proteoglycan would weaken close contact adhesion processes and be an important parameter in cellular movement. A better understanding of these dynamic processes will be required to clarify the mechanisms for the making and breaking of adhesive contacts of fibroblasts with this model extracellular matrix.

Registry No. Hyaluronic acid, 9004-61-9; chondroitin sulfate, 9007-28-7; heparan sulfate, 9050-30-0.

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Hydrophobic Labeling of (Na^+,K^+)-ATPase: Further Evidence That the β Subunit Is Embedded in the Membrane Bilayer†

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ABSTRACT: *O*-Hexanoyl-3,5-diiodo-*N*-(4-azido-2-nitrophenyl)tyramine has been used after photochemical conversion into the reactive nitrene to label (Na^+,K^+)-ATPase from *Bufo marinus* toad kidney. Immunochemical evidence indicates that the reagent labels both subunits of the enzyme in partially purified form as well as in microsomal membranes. These results support the view that the glycoprotein subunit, like the catalytic subunit, possesses hydrophobic domains by which it is integrated into the plasma membrane.

Sodium- and potassium-dependent adenosine triphosphatase [(Na^+,K^+)-ATPase], which mediates the active transport of Na^+ and K^+ ions across the plasma membrane of animal cells, is composed of two subunits. The larger polypeptide (α subunit) spans the membrane and requires phospholipids for expression of the catalytic activity (Kyte, 1975; Roelofsen & Schatzmann, 1977). In contrast, the relationship of the glycosylated polypeptide (β subunit) with the lipid bilayer and the function of the β subunit are not known. In order to understand the assembly and the function of these subunits at the molecular level, it is important to know their topographical distribution and their orientation in the membrane as well as their modes of attachment with the lipid bilayer.

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