Biochemical Analysis of *Caenorhabditis elegans* Surface Mutants

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Abstract: A collection of *Caenorhabditis elegans* mutants that show ectopic surface lectin binding (Srf mutants) was analyzed to determine the biochemical basis for this phenotype. This analysis involved selective removal or labeling of surface components, specific labeling of surface glycans, and fractionation of total protein with subsequent detection of wheat germ agglutinin (WGA) binding proteins. Wild-type and mutant nematodes showed no differences in their profiles of extractable surface glycoproteins or total WGA-binding proteins, suggesting that the ectopic lectin binding does not result from the novel expression of surface glycans. Instead, these results support a model in which ectopic lectin binding results from an unmasking of glycosylated components present in the insoluble cuticle matrix of wild-type animals. To explain the multiple internal defects found in some surface mutants, we propose that these mutants have a basic defect in protein processing. This defect would interfere with the expression of the postulated masking protein(s), as well as other proteins required for normal development.

Key words: biochemical analysis, *Caenorhabditis elegans*, glycoproteins, lectin, nematode, surface mutant.

The nematode cuticle is a complex, acellular, primarily collagenous structure essential for locomotion, maintenance of morphology, and protection against the environment (Bird and Bird, 1991). In zooparasitic nematodes, the cuticle is a major site of recognition and attack by the host immune system, and similar surface reactions may play a part in phytoparasite-host recognition or attachment of pathogens to nematodes (Jansson, 1994; Tunlid et al., 1992). Cuticle surface proteins have been described that are hypothesized to be involved in cuticle maintenance, organismal nutrition by absorption across the cuticle, or active enzymatic immune evasion (Maizels et al., 1993). Many of these proteins are modified by asparagine- (N-) linked or serine/threonine- (O-) linked glycans (Maizels et al., 1989; Page

and Maizels, 1992). The role of these modifications is unknown, but they represent a potential chemotherapeutic or vaccine target. Analysis of protein glycosylation and cuticle biosynthesis in parasitic species is compromised by scarcity of material. The cuticle of the free-living species *Caenorhabditis elegans* provides an excellent model to examine the genetics and biochemistry of the construction and maintenance of the nematode cuticle (Politz and Philip, 1992). The morphological conservatism of the phylum suggests that findings on the *C. elegans* cuticle can be extended to those of parasitic species. To this end, *C. elegans* strains harboring mutations that alter surface binding of antisera or lectins have been isolated (the Srf phenotype) (Link et al., 1992; Politz et al., 1987; Politz et al., 1990). These *srf* loci define a set of functions required for normal cuticle synthesis that do not result in gross morphological abnormality or significant lethality when mutated.

Link et al. (1992) described the isolation of 45 mutant strains of *C. elegans* that exhibit altered surface binding of the lectins wheat germ agglutinin (WGA; recognizing N-acetyl-glucosamine) and soybean agglutinin (SBA; recognizing N-acetyl-galactosamine). These recessive mutations define six genes: *srf-2*, *srf-3*, *srf-4*, *srf-5*, *srf-8*, and *srf-9*. The *srf-2* and *srf-3* loci were independently described by Politz et al. (1987, 1990) using a stage-

specific, surface-reactive antibody screen. The genes can be placed in two classes based on the phenotypes of the mutant animals. The "simple" Srf mutants (*srf-2*, *-3*, and *-5*) are essentially wild type except for the lectin-binding phenotype. Whereas live, wild-type nematodes bind WGA weakly, and only at the male copulatory bursa and the hermaphrodite vulva, these mutants show binding over the whole cuticle surface. The pleiotropic Srf mutants (*srf-4*, *-8*, and *-9*) have multiple defects in addition to the lectin phenotype. These pleiotropic defects include uncoordinated movement, abnormal egg-laying, vulval cell fate determination alterations, and defective morphogenesis of the male copulatory bursa. Analysis at the cellular level reveals additional defects in gonadal distal tip cell migration and axonal morphology.

We are interested in the biochemical and structural bases of the changes resulting from the Srf mutations. At least two hypotheses can be proposed: (i) that the *srf* loci correspond to genes controlling glycosylation or another facet of post-translational modification of cuticle proteins, and (ii) that the Srf phenotype results from the loss of a surface structure that otherwise would mask determinants present in both wild-type and mutant cuticles. Although these hypotheses are not exclusive, we examined the localization of cuticle glycoproteins and their biochemistry in the Srf mutants to address which is more likely. The surface of nematodes can be broken down ultrastructurally into a series of distinct layers (Bird and Bird, 1991), and these can be examined biochemically and in vivo by specific fractionation techniques (Blaxter, 1993; Maizels et al., 1993).

MATERIALS AND METHODS

Caenorhabditis elegans strains and culture: Strains used in this work were described by Link et al. (1992). We chose representative pleiotropic (CL264 *srf-8(dv-38)*V) and nonpleiotropic (CL261 *srf-5(ct115)*X; *him-5(e1490)*V) strains to compare with the wild type and subjected them to a variety of bio-

chemical analyses to define more closely the relationship between cuticle structure, composition, and the Srf phenotype before extending findings to other alleles and other Srf loci, including CL183 *srf-4* (*ct109*); *him-5(e1490)*V. Culturing and handling were as described previously (Brenner 1974: Wood, 1988). All nematodes were grown and maintained at 20 °C. Large synchronous cultures of *C. elegans* were prepared from hypochlorite-cleaned eggs (Epstein et al., 1996).

Fluorescent labeling of live nematodes: Wheat germ agglutinin coupled to fluorescein isothiocyanate (WGA-FITC) labeling of live, adult hermaphrodite nematodes was carried out as previously described (Link et al., 1992). Specificity was shown by abrogation of labeling in the presence of 200 mM N-acetyl glucosamine. We examined the involvement of the surface coat and the epicuticle in the WGA-binding phenotype of the Srf mutants by removing these structures from intact nematodes with graded ethanol washes (Page et al., 1992). For comparison of treated and untreated nematodes, the nuclear DNA stain, *bis*-benzimide, was included at 1 µg/ml in the WGA staining step of the ethanol-treated group to visibly tag the nematodes. To examine the involvement of the surface coat and epicuticle in lectin binding, nematodes were preincubated in ethanol (10%, 50%, and 100%) before processing for WGA-FITC labeling. The surface or epicuticular lipid of the nematode cuticle was directly visualized with 5-N-(octadecanoyl) aminofluorescein (AF18; Molecular Probes, Eugene, OR). Adult hermaphrodites were labeled for 10 minutes at 25 °C on teflon-coated slides by incubating them in 10 µg of AF18 diluted in 1 ml of sterile M9 buffer (Epstein et al., 1996). The nematodes were washed in M9 for 10 minutes before mounting in 0.1% sodium azide in M9. Nematodes were observed with epifluorescence optics.

Labeling of nematode carbohydrates and fractionation of proteins: The following experiments were designed to determine differences in the protein and glycoprotein content of wild-type and Srf nematodes. Nematodes were sequentially fractionated to

obtain nonionic detergent (NonidetP-40, NP-40)-soluble proteins, ionic detergent (sodium dodecyl sulphate, SDS)-soluble proteins, and 2-mercaptoethanol (2ME)-soluble collagens. Each strain was prepared twice, and fractions were analyzed by SDS-PAGE and lectin blotting in triplicate. For each strain analyzed, 100,000 young adult nematodes were washed from plates in M9 buffer and repeatedly pelleted in a low-speed centrifuge to remove bacteria and other contaminants. The nematodes were resuspended in 300 μl of 1% NP-40 in phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4) with protease inhibitors (PI: 1 mM EDTA, 1 mM EGTA, 0.1 mM pepstatin, 1 mM PMSF) and sonicated on ice until all the nematodes were broken open (assessed by microscopy). The sonicate was rocked at 20 °C for 30 minutes, and insoluble material was pelleted by centrifugation at $12,000g$ for 15 minutes. The supernatant was removed and saved, then the pellet was resuspended in 500 μl of 1% SDS in PBS-PI and sonicated for 5 minutes on ice. This suspension was then boiled for 10 minutes and rocked at 20 °C for 10 minutes before removal of insoluble material by centrifugation as before. The pellet was resuspended in 100 μl 1 NP-40, 2% 2ME in PBS-PI and boiled for 10 minutes. After rocking at 20 °C for 30 minutes, insoluble material was pelleted as before. The three supernatants and the insoluble residue were stored at −20 °C. Protein was quantified with the bicinchoninic acid assay (Pierce Chemical, Rockford, IL).

For hydrazide labeling of surface carbohydrate components, 1,000 adult hermaphrodites were cleaned by centrifugation and resuspended in 40 μl of 0.1 M sodium acetate buffer, pH 5.5. Five mM sodium periodate was added, and the reaction was incubated at 20 °C for 10 minutes in the dark. The periodation was stopped by the addition of 5 mM sodium bisulfite and incubation for an additional 5 minutes. Ten μl of digoxigenin-3-O-succinyl-e-aminocaproic acid hydrazide (DIG-hydrazide; 0.75 mg/ml in dimethyl formamide) (Boehringer Mannheim, Indianapolis, IN) was added and the labeling re-

action incubated at 20 °C for 1 hour. The reaction was terminated by rinsing the nematodes three times in excess M9 buffer. Visualization of digoxygenin (DIG)-labeled surface glycan components on intact nematodes was achieved by incubation in anti-DIG antibody (1:100 dilution in PBS with 2% polyvinylpyrrolidone [PVP]; Boehringer Mannheim, Indianapolis, IN) for 1 hour at 20 °C. After rinsing three times for 5 minutes in PBS with 0.05% Tween 20, the nematodes were incubated in anti-sheep-FITC conjugate (1:1,000 in PBS with 2% PVP) (Vector Laboratories, Burlingame, CA) for 1 hour at 20 °C. The nematodes were washed three times in PBS Tween before fluorescence microscopy. Soluble labeled proteins were released by boiling DIG-labeled nematodes in 100 μl SDS-PAGE sample buffer (0.25 M Tris HCl, pH 6.8, 8% SDS, 40% glycerol, 20% 2ME, 0.1% bromophenol blue) for 10 minutes.

SDS-PAGE and detection of glycoproteins on western blots: To visualize N-acetyl glucosamine-containing glycoproteins, immobilized proteins were probed using WGA conjugated to DIG. DIG label was used in preference to biotin because of the presence of major endogenous biotin-containing proteins in *C. elegans*, which confound analysis with avidin conjugates (M. Blaxter, unpubl.). Discontinuous SDS-PAGE (Laemmli, 1970) was performed on minigels (Mighty Small II, Hoeffer, San Fernando, CA). Gels were stained for protein with Pro-Blue (Integrated Separation Systems, Natick, MA) or were Western-blotted onto polyvinyldifluoridine (PVDF) membranes (Millipore, Bedford, MA). Blots were rinsed in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5), and blocked in 2% PVP in the same buffer for 2 hours at 20 °C. After rinsing in maleate buffer, blots of unlabeled protein extracts were incubated in DIG-labeled WGA (1 mg/ml in blocking buffer) (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 20 °C. The blots were then washed three times for 10 minutes each in maleate buffer with 0.05% Tween 20 and incubated in anti-DIG antibody conjugated to alkaline phosphatase (150 U/ml calf intestinal alka-

line phosphatase; Boehringer Mannheim, Indianapolis, IN) diluted 1:5,000 in maleate buffer for 1 hour at 20 °C. The blots were washed as before in maleate buffer with 0.05% Tween 20 before colorimetric development with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (X-phosphate and NBT; Boehringer Mannheim, Indianapolis, IN). DIG-hydrazide-labeled extracts were analyzed on SDS-PAGE, blotted, and developed with anti-DIG-AP as above.

Protease and glycanase treatment: To demonstrate that the major Mr 115 kDa WGA-binding species is a glycoprotein, NP-40 fractions were treated with the non-specific protease Proteinase K, and the glycosidase N-glycanase, which cleaves asparagine-linked oligosaccharides from glycoproteins. Unlabeled NP-40 extract (50 µg protein) from each strain was treated with 2 µg/ml Proteinase K for 30 minutes at 37 °C before analysis with SDS-PAGE and lectin blotting. Unlabeled NP-40 extract (50 µg protein) from each strain was placed in a total volume of 20 µl containing 0.17% SDS and 0.1 M 2ME, and boiled for 3 minutes. The reaction mixture was 0.55 M sodium phosphate, pH 6.8, and 1.25% NP-40, and 2.4 µl of native N-glycanase (10 U/ml; Genzyme, Cambridge, MA) was added to make a final volume of 30 µl. The reaction was allowed to proceed overnight at 37 °C, and samples were analyzed with SDS-PAGE and lectin blotting.

Analysis of insoluble components: To determine if DIG-tagged glycoconjugates from the surface-labeling experiments were not solubilized, the insoluble pellets remaining after the DIG-hydrazide surface-labeling experiment were probed for the presence of DIG-labeled sugars using an anti-DIG primary antibody and a fluorescent secondary antibody. The residual material from the fractionation of unlabeled nematodes was stained with WGA-FITC (diluted 1:50; 5 mg/ml) (Vector Laboratories) in M9 buffer for 1 hour at 20 °C. The material was rinsed three times in M9 before transfer to microscope slides and viewing with epifluorescence optics. Residual material from DIG-hydrazide

labeling was stained with anti-DIG antibodies following the protocol used for whole nematodes as described above.

RESULTS

Nematode surface: Treatment of nematodes with ethanol did not render the cuticles of adult, wild-type nematodes WGA-positive but did result in binding of the lectin to structures identified as the amphids, the excretory pore (and duct in some specimens), and phasmids (Fig. 1A,B). Faint WGA-binding to many internal structures also was observed. The amphids, excretory pore, and phasmids of Srf nematodes also were labeled (Fig. 1C,D). Ethanol stripping did not abrogate WGA-binding to Srf mutants and, indeed, enhanced the cuticular labeling phenotype (Fig. 1E-J). After ethanol treatment, strong labeling of nuclei was observed with 1% of the standard concentration (i.e., 0.01 mg/ml) of *bis*-benzimide reagent, suggesting that a barrier to *bis*-benzimide uptake had been removed. No difference in intensity or extent of staining with AF18 was observed among *srf-5*, *srf-8*, and wild-type adult nematodes.

Surface-exposed glycoconjugates: DIG-hydrazide labeling was employed to label surface-exposed reducing sugars. To confirm the surface specificity of this technique, DIG-labeled components were visualized with anti-DIG antibody and anti-immunoglobulin-FITC conjugate on intact nematodes. The nematodes remained viable until the addition of dimethyl formamide, and remained intact thereafter. Parallel labeling of nematode homogenates suggested that labeling was limited to a surface subset of glycoconjugates. A qualitative difference in surface staining was observed between Srf and wild-type nematodes. Staining with DIG-hydrazide was evident over the entire surface of *srf-5* and *srf-8* adults, whereas the wild type exhibited staining only around the head, tail, and vulva. This labeling pattern strongly resembled the WGA-binding phenotypes.

Wild type, *srf-5*, and *srf-8* nematodes surface-labeled with DIG-hydrazide were com-
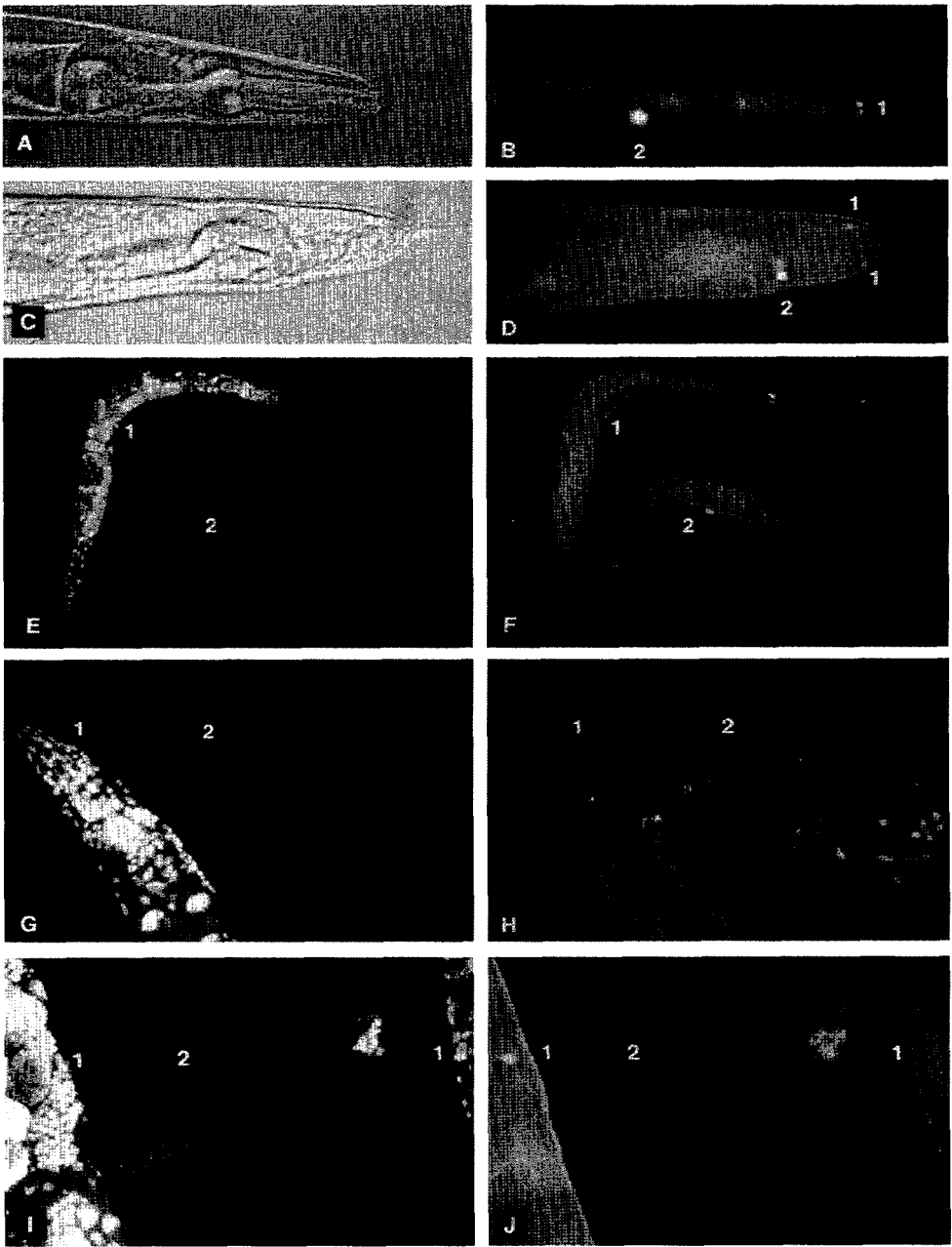
FIG. 1. The Srf phenotype and ethanol stripping of superficial layers A, B) Head and pharynx of an N2 (wild-type) adult hermaphrodite, treated with 100% ethanol for 10 seconds and labeled with WGA-fluorescein. A) Nomarski optics. B) Fluorescence optics. The amphids (1) and excretory pore (2) are stained with WGA, but the cuticle is not labeled. C, D) Tail of an *srf-3* adult hermaphrodite, treated with ethanol for 10 seconds and labeled with WGA-fluorescein. C) Nomarski optics. D) Fluorescence optics. The cuticle surface, phasmids (1), and anal slit (2) are stained with WGA. E, F) Two *srf-8* adult hermaphrodites either treated with ethanol for 10 seconds and then labeled with *bis*-benzimide a fluorescent nuclear stain (1) and WGA-fluorescein, or incubated only in WGA-fluorescein (2). E) Fluorescence optics, blue (*bis*-benzimide) channel. F) Fluorescence optics, green (fluorescein) channel. The cuticles of both animals are stained with WGA. G, H) Two *srf-4* adult hermaphrodites either treated with ethanol for 10 seconds and then labeled with *bis*-benzimide and WGA-fluorescein, or incubated only in WGA-fluorescein (2). G) Fluorescence optics, blue (*bis*-benzimide) channel. H) Fluorescence optics, green (fluorescein) channel. The cuticles of both animals are stained with WGA. The excretory pore and duct are labeled in the ethanol-treated nematode. I, J) *srf-5* adult hermaphrodites either treated with ethanol for 10 seconds and then labeled with *bis*-benzimide and WGA-fluorescein (1), or incubated only in WGA-fluorescein (2). I) Fluorescence optics, blue (*bis*-benzimide) channel. J) Fluorescence optics, green (fluorescein) channel. The cuticles of both treated and untreated animals are stained with WGA.

pared and a labeled band of Mr 40 kDa was observed in each strain, but not in unlabeled extracts. No differences in surface glycoprotein components were observed in Srf and wild-type nematodes (Fig. 2). No qualitative differences in surface lectin binding and DIG labeling between wild-type and Srf nematodes was seen by Western blot analysis of soluble components.

Soluble cuticle matrix components and somatic glycoproteins: Direct staining of gels did not reveal any reproducible changes in the overall protein profile in any of the protein fractions when comparing Srf mutants and the wild type. Two WGA-binding species were consistently observed (Fig. 3). The major WGA-binding species (Mr 115 kDa) was found in the NP-40 extract. The SDS fraction also contained some of the Mr 115 kDa species. A second WGA-binding species (Mr 210 kDa) was found predominantly in the SDS fractions. Both of these species were present in wild-type and Srf nematodes, at comparable levels. No WGA-binding species were observed in the 2ME-extracted protein fraction. Subsequent analysis of *srf-2* (*ct104*), *srf-3* (*ct107*), *srf-4* (*ct109*), and *srf-9* (*dv4*) revealed the same WGA-binding pattern (data not shown). Both proteinase K and N-glycanase treatments suppressed detection of the 115 kDa species with WGA, indicating that it is a protein with N-linked glycan sidechain(s).

Insoluble cuticle components: WGA-FITC bound strongly to the SDS-2ME residual material from all strains (data not shown). The insoluble material from all strains tested showed the same amount of staining. This result indicated that many DIG-hydrazide-labeled sugars were present but insoluble in wild-type and mutant nematodes.

DISCUSSION

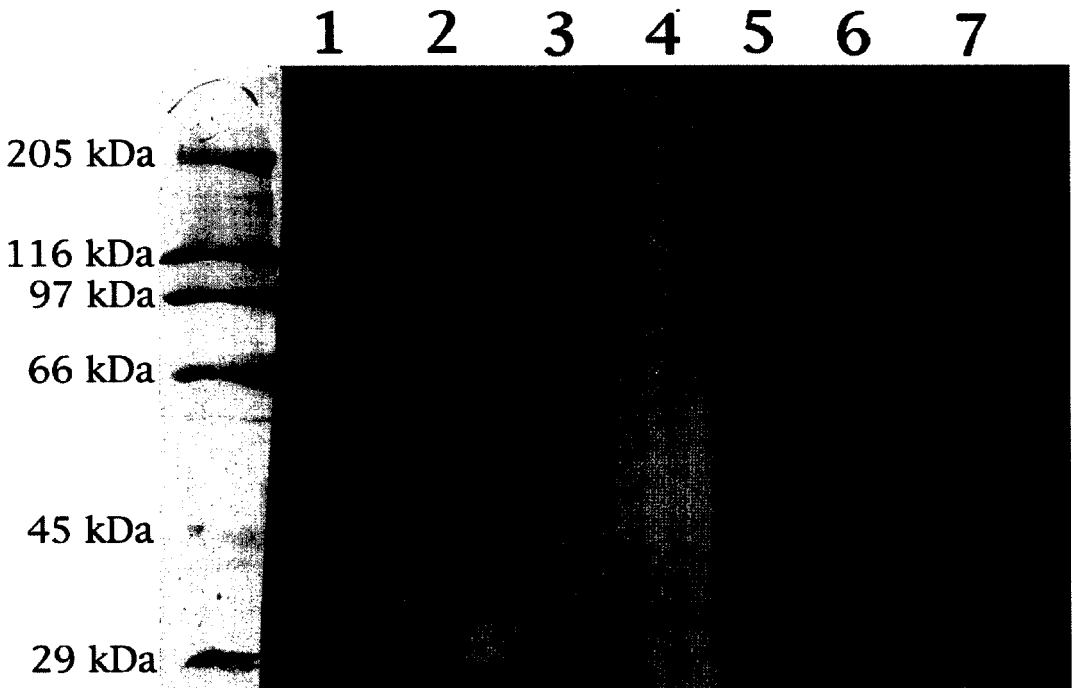We have proposed two models to explain the nature of the underlying defect(s) re-

FIG. 2. Direct carbohydrate labeling of *Caenorhabditis elegans* surface carbohydrate. Live nematodes were labeled with digoxigenin-hydrazide. Surface-labeled animals were solubilized with SDS/2ME and the proteins were fractionated by SDS-PAGE, blotted, and probed with an anti-digoxigenin antibody. Lanes: 1 = N2, 2 = *srf-5*, 3 = *srf-8*, 4 = N2, NP-40 extracted proteins, 5 = *E. coli*, 6 = unlabeled, solubilized N2 nematodes, 7 = N2 eggs. A single species of approximately Mr 40 kDa (arrow) was identified in all labeled nematode strains analyzed.
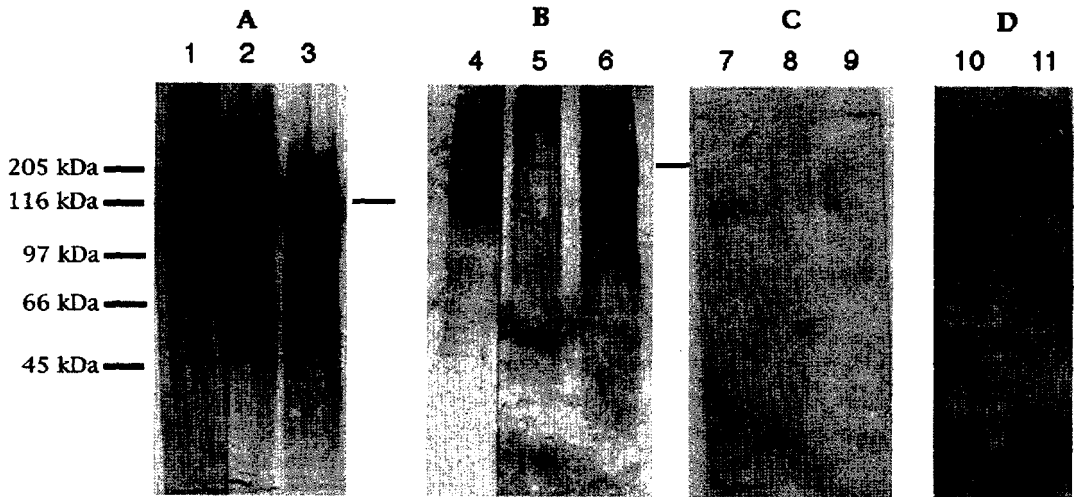
FIG. 3. Soluble lectin-binding components from *Caenorhabditis elegans*. Sequentially extracted protein preparations were fractionated on SDS-PAGE, blotted, and probed with WGA-digoxigenin as described in the text. Approximately 50 µg of protein was loaded in each lane. A) NP 40 extract. Lane 1 = N2, 2 = *srf-5*, 3 = *srf-8*. B) SDS extract. Lane 4 = N2, 5 = *srf-5*, 6 = *srf-8*. C) NP 40/2ME. Lane 7 = N2, 8 = *srf-5*, 9 = *srf-8*. D) Controls. Lane 10 = *E. coli* NP 40/SDS extracted proteins, 11 = carbonic anhydrase (WGA positive control).

vealed by Srf mutants. The first asserts that the Srf phenotype results from the unmasking of WGA-binding targets present but inaccessible in wild-type cuticles (Politz et al., 1990). This model arises from the observation that the epitope recognized by the antiserum used to define some *srf-2* and *srf-3* alleles was generated by immunization with wild-type cuticle preparations. In these experiments, an adult-specific, anti-cuticle antiserum was shown to bind to the surface of fourth-stage juveniles (J4) of *srf-2* and *srf-3* nematodes. The Srf phenotype was explained by proposing that a masking structural layer had been ablated (Politz et al., 1990).

The second model asserts that the defects are due to protein and glycoprotein processing. This model was based on the observation of lectin binding to the surface of Srf mutants and the existence of a class of pleiotropic Srf mutants with additional defects in internal structures (Link et al., 1992). Biosynthetic processes potentially implicated include protein glycosylation, protein targeting or sorting, membrane assembly (including protein attachment to membranes), and extracellular assembly of protein complexes. The two hypotheses can be reconciled if the

missing layer is reduced to molecular terms and modeled as a terminal glycosylation processing step that either fails to remove or ectopically adds a WGA-reactive sugar moiety to surface-exposed structures. This model predicts that the pleiotropic mutants will express somatic defects in processing of (glyco)proteins that may be more extensive than those seen in the simple mutants.

We first examined the surface structures of the nematodes directly by staining of treated and untreated intact adults with surface-reactive probes. Live–staining of adult nematodes with the lipophilic probe AF18 (Kennedy et al., 1987; Proudfoot et al., 1990) indicated that the epicuticle is present in the Srf mutants. Surface labeling with IODOGEN revealed minor changes in lipid composition of the epicuticle (Blaxter, 1993), but the relationship of these changes to the Srf phenotype is unclear. Stripping of the epicuticular lipid and surface coat with organic solvent (Page et al., 1992) did not significantly affect wild-type or Srf staining patterns. We conclude that the surface coat and epicuticular lipids are not the structural sites of the lectin-binding phenotype and that they are not the masking layer proposed by the first model, although they may im-

pede access of the lectin to the target site(s). Ethanol treatment does not remove the major surface-located protein, surfin (Blaxter, 1993).

The Srf phenotype could result from the expression of novel glycoconjugates at the surface. Glycoconjugates would be expected to occur in the surface coat and in the matrix of the cuticle. Although direct labeling of surface-exposed carbohydrates with DIG-hydrazide identified an Mr 40 kDa glycoprotein not previously identified with radioactive surface labeling, no differences were observed in the expression of this or other DIG-labeled proteins between wild-type and Srf nematodes. The major IODOGEN-labeled proteins are not tagged with DIG-hydrazide, confirming that they are not glycosylated (Blaxter, 1993). DIG-hydrazide can label multiple glycosylated proteins in detergent extracts of nematodes, including one with the same mobility as the WGA-binding Mr 115 kDa protein. In intact nematodes examined by fluorescence microscopy, more intense DIG-hydrazide staining of Srf nematodes was observed, suggesting that the reagent had greater access to the labeled species, but these species were not solubilized despite the relatively harsh extraction procedure utilized.

We looked for alterations in the WGA-binding glycoprotein content of the mutants using protein fractionation and lectin blotting techniques. We identified two major WGA-binding species in these studies: an abundant NP-40 extractable species of Mr 115 kDa and a less abundant Mr 210 kDa species found predominantly in the SDS fraction. The identity of these proteins is not known. While the 115 kDa glycoprotein had a mobility similar to one of the major yolk proteins of *C. elegans,* its migration as a diffuse band on SDS-PAGE differs from the tight banding seen for the yolk protein (Sharrock, 1983). The 2ME protein fraction, primarily representing cuticle collagens, did not bind WGA in lectin blotting experiments. Becker et al. (1993) reported a detection sensitivity of approximately 65 ng of labeled glycoprotein in lectin blotting experiments. Based on this sensitivity level, it is

not likely that we would detect a glycoprotein that was less than 0.1% of the total protein. Subtle differences in glycosylation of the Srf mutants thus may not be detectable with the methods employed.

The insoluble components of the cuticle have been termed cuticlin. Where examined in other nematodes such as *Ascaris suum,* cuticlins comprise a diverse set of protein and carbohydrate components extensively cross-linked by non-peptide bonds (Betschart and Wyss, 1990; Fetterer et al., 1993; Fujimoto and Kanaya, 1973). Intense lectin staining was observed in the insoluble material from Srf mutants and wild-type nematodes, indicating that one or more WGA-binding species cannot be solubilized under our conditions. Similar analysis of the insoluble components from DIG surface-labeled nematodes with a fluorescein-tagged, secondary antibody confirms the presence of insoluble glycosylated components on or near the surface of wild-type and Srf nematodes. In IODOGEN labeling of live nematodes, a significant proportion of the incorporated label (~30%) also remained insoluble (Blaxter, 1993). We thus conclude that the WGA-binding targets seen in lectin labeling of live Srf nematodes most likely are insoluble components of the cuticle matrix and that these targets are present in both wild-type and Srf mutant nematodes. Of course, there may be differences in the structure of these insoluble glycans between the mutant and wild-type nematodes that our analyses could not detect. Lectin binding to ultrathin sections of cuticles of many parasitic nematodes has shown that glycans are found throughout the cuticle matrix and are often concentrated in particular zones, including the cortical zone that underlies the epicuticle (Rudin, 1990). This zone contains material that remains insoluble after 2ME extraction. Biochemical analysis of 2ME-extracted cuticle remnants from parasitic nematodes also has shown the presence of glycans in the cuticlin fraction. The Srf phenotype would thus seem to result from an increase in accessibility of these matrix components to the extrinsic reagents. Genes encoding components of *C. elegans* cuticlin

have been isolated (Lassandro et al., 1994; Sebastiano et al., 1991). These genes do not have consensus N-glycosylation sites, suggesting that if they are glycosylated, other linkages are used.

We suggest that the weight of evidence favors the unmasking hypothesis as an explanation for the Srf cuticle phenotype. This hypothesis is further supported by analysis of recently isolated extragenic suppressors of *srf-5* (Enyeart, Blaxter, and Link, unpubl.). These suppressor mutations, which block the ectopic lectin binding of *srf-5* mutants, also restore surfin to the cuticle surface. We have been unable to conclusively identify the masking component.

If no obvious glycosylation defects exist in Srf mutants, how can the pleiotropic defects of some of the Srf mutants be explained? Surfin is a hydrophobic molecule that becomes hydrophilic upon treatment with reducing agents (Blaxter, unpubl.). One mechanism that could explain these properties is that surfin carries a thioester-linked lipid anchor that in situ attaches it to the epicuticle. The pleiotropic Srf mutants may have defects in hypodermal cells that prevent addition of this anchor and, thus, ablate the surface expression of surfin. It is noteworthy in this context that the Srf mutants also have changes in their surface lipid profiles. Lipid modifications are essential for many cellular processes, and the pleiotropic defects seen in *srf-4*, *srf-8*, and *srf-9* nematodes may result from such an extensive disruption of protein sorting, targeting, and secretion.

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