A Novel Giant Secretion Polypeptide in *Chironomus* Salivary Glands: Implications for Another Balbiani Ring Gene

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ABSTRACT *Chironomus* salivary glands contain a family of high M_r (~1,000 × 10^3) secretion polypeptides thought to consist of three components: sp-Ia, sp-Ib, and sp-Ic. The use of a new extraction protocol revealed a novel high M_r component, sp-Id. Results of a survey of individual salivary glands indicated that sp-Id was widespread in more than a dozen strains of *C. tentans* and *C. pallidivittatus*. Sp-Id was phosphorylated at Ser residues, and a comparison of cyanogen bromide and tryptic peptide maps of ^{32}P-labeled polypeptides suggested that sp-Ia, sp-Ib, and sp-Id are comprised of similar but nonidentical tandemly repeated amino acid sequences. We concluded that sp-Id is encoded by an mRNA whose size and nucleotide sequence organization are similar to Balbiani ring (BR) mRNAs that code for the other sp-I components. Furthermore, parallel repression of sp-Ib and sp-Id synthesis by galactose led us to hypothesize that both of their genes exist within Balbiani ring 2.

Unusual structural proteins are secreted by a pair of simple yet highly specialized salivary glands in aquatic larvae of the midge, *Chironomus* (for reviews, see references 17 and 18). The perimeter of each gland consists of 30–40 secretory cells that surround a central lumen. A limited number of tissue-specific secretion polypeptides are synthesized in these cells, secreted into the lumen of the gland, and then passed through a salivary duct which leads to the mouth of the larva. Although secretion polypeptides are initially soluble in the glandular lumen, as they pass through the salivary duct, they undergo a dramatic transformation that results in their polymerization into a long, elastic silken fiber. This salivary fiber is vitally important for filter feeding (for review, see reference 38).

Although little is known about the polymerization of *Chironomus* secretion, various biochemical properties of its constituent polypeptides are known. For example, there is a family of high M_r (~1,000 × 10^3) secretion polypeptides (sp-I)[1] that contains several components (see Materials and Methods). Electrophoretic analysis of secretion extracted from the lumen of salivary glands indicates that 4th instar larvae typically synthesize two sp-I components: sp-Ia and sp-Ib (19, 30). The addition of galactose or certain other monosaccharides to larval culture medium stimulates synthesis of an additional sp-I component (sp-Ic) and simultaneously inhibits synthesis of sp-Ib (12, 13). The simple amino acid composition (16, 20) and peptide maps (19, 20, 30, 33) of secretion suggest that the primary structure of its component polypeptides consists of arrays of repeated amino acid sequences especially rich in basic residues (~25% Arg + Lys). Finally, sp-Ia and sp-Ib are glycosylated (20) and phosphorylated (15).

In this paper, we describe the discovery of a fourth giant secretion polypeptide (sp-Id) in salivary glands of *Chironomus* larvae. This novel sp-I component was routinely observed when the extraction and electrophoretic separation of salivary gland protein was done under carefully prescribed conditions. To determine whether or not this novel sp-I component was strain-specific, numerous European and North American strains of *C. tentans* and *C. pallidivittatus* were examined for their content of sp-Id. The phosphoamino acid content, phosphopeptide fingerprints (derived by cyanogen bromide or trypsin cleavage), and galactose-induced alterations in the electrophoretic display of sp-I components were compared. These data implied that sp-Id was encoded by a fourth large gene comprised of tandemly repeated protein-coding sequences similar to the Balbiani ring (BR) genes BR1, BR2,

[1] *Abbreviations used in this paper:* BR, Balbiani ring; Gdn-HCl, guanidine hydrochloride; Met, methionine; sp-I, family of high M_r secretion polypeptides.

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and BR6 which encode the other sp-I components (sp-Ia, sp-Ib, and sp-Ic, respectively). Furthermore, we will propose that one BR may possibly contain two of these genes.

MATERIALS AND METHODS

Nomenclature: Participants at an international workshop on "The Structure and Evolution of Balbiani Ring Genes" held in Gatersleben, GDR in October, 1983 agreed to a standard convention that will be used to refer to *Chironomus* salivary gland secretion polypeptides and BR genes that encode some of them. sp-I contains three previously described components: sp-Ia, sp-Ib, and sp-Ic (12). In this paper, we describe a fourth component of this family, designated as sp-Id. The following are designations used to describe cloned repeats from BR genes: BR1α (6, 42), BR2α (36), BR2β (BR2min in reference 8; pCt7 in reference 41), and BR6α (26).

Raising Larvae: Larvae were normally raised at 16°–18°C in tubs containing 6 liters of aerated 0.04% NaCl as their culture medium (7). Beermann's (4) procedure for galactose-induction experiments was modified as follows. Larvae were placed in 6 liters of fresh culture medium containing 0.5% galactose and were provided initially with a minimum amount of food. During the duration of the experiment, the galactose-containing culture medium was not changed. The motivation behind these modifications was to treat larvae exposed to galactose in a manner as parallel as possible to their siblings in control cultures. The results of these modifications were (*a*) a significantly increased percentage of viable larvae, (*b*) comparable numbers of normal and galactose-treated larvae continued to develop into pupae and adults, and (*c*) an increase in the time required before galactose-induced alterations of the electrophoretic display of sp-I components could be observed.

Radioisotopic Labeling of Salivary Gland Protein: Salivary gland protein was isotopically labeled in organ culture in modified Cannon's medium (25) containing [^{35}S]methionine ([^{35}S]Met) (1,390 Ci/mmol; 1 mCi/ml) or ^{32}P-orthophosphate (5 mCi/ml) in Met- or phosphate-free (25 mM HEPES, pH 7.2) medium, respectively. In some experiments, salivary gland protein was labeled in vivo by the addition of [^{35}S]Met (50 μCi/ml) or ^{32}P-orthophosphate (200 μCi/ml) to 10 ml of culture medium containing as many as 20 free-swimming larvae.

Extraction of Salivary Gland Protein: Between 1 and 20 salivary glands were added to a 1.5-ml microcentrifuge tube containing 0.1 ml of ice-cold extraction medium containing 6 M guanidine hydrochloride (Gdn-HCl), 40 mM Tris-HCl, pH 8.8, 20 mM EDTA, 100 mM β-mercaptoethanol (or dithiothreitol), and 1 mM phenylmethylsulfonyl fluoride. Glands were vortexed several times and allowed to dissolve for up to 1 h on ice, then 10 μl of 1 M iodoacetamide was added, the samples mixed, and incubated in the dark for 1 h at room temperature. Extracts were centrifuged at 12,000 *g* for 5 min to pellet undissolved debris. The supernatant was carefully removed, transferred to another tube, and protein was precipitated overnight at −20°C by the addition of 9 vol of 80% acetone. Protein was pelleted by centrifugation at 12,000 *g* for 3 min, lightly air-dried, and resuspended in 30 μl of 10 mM Tris-HCl, pH 7.2. 1 μg of RNase A was added, samples were incubated for 10 min at 37°C, mixed with an equal volume of 2× sample application buffer containing SDS (23), and boiled for 3–5 min. Similar schemes were attempted with extraction media containing 8 M urea (16), 2.5% SDS (30), and 5 M guanidine thiocyanate (9); however, neither the quantity nor quality of the yield of high molecular weight secretion polypeptides was as reproducible as with Gdn-HCl.

For some experiments, protein was extracted from various components of a salivary gland (i.e., the lumen or secretory cells). This was done by fixing the glands in 70% ethanol, dissecting the components with a de Fonbrune micromanipulator (25), and dissolving them in Gdn-HCl extraction medium as described above.

Gel Electrophoresis: Salivary gland polypeptides were resolved by polyacrylamide gel electrophoresis in the presence of SDS as described by Laemmli (23) and modified as follows: Each 12 × 14 cm, 1.5-mm-thick slab gel contained a 3–20% (wt/vol) concave exponential polyacrylamide gradient. Acrylamide solutions were diluted from a stock of 30% acrylamide, 0.8% *N,N'*-methylene-bis-acrylamide. A gradient marker (Hoeffer Scientific Instruments, San Francisco, CA) was used in which the reservoir contained 25 ml of 3% acrylamide, and the mixing chamber (with a fixed volume of 6.26 ml) initially contained 20% acrylamide plus 20% (vol/vol) glycerol to stabilize the gradient as it was pumped between the gel casting plates. Each solution of polyacrylamide also contained 0.1% TEMED and 0.7% ammonium persulfate. The polymerization time for these gels varied more than 10-fold depending upon the commercial source of acrylamide and catalysts. Therefore, it was critical to monitor polymerization of a small aliquot before constructing one of these gradient gels. Protein samples in 25–60 μl of 1× sample application buffer were electrophoresed at a constant current of 30 mA per gel for 3–4 h. Gels were stained with silver nitrate (Bio-Rad Laboratories, Richmond, CA) and photographed. Gels containing ^{32}P-labeled protein were directly exposed to x-ray film, whereas gels with ^{35}S-labeled protein were dried before autoradiography.

Identification of Phosphoamino Acids: Samples of salivary gland protein labeled with ^{32}P in vivo or in organ culture were electrophoresed in exponential polyacrylamide gradient gels and secretion polypeptides located by autoradiography of the wet gel. The desired bands were cut out and electroeluted in electrode buffer (23) using an Isco model 1750 sample concentrator (Isco, Inc., Lincoln, NE). Radioactive protein was precipitated at −20°C with acetone. After centrifugation at 12,000 *g* for 15 min, the precipitates were dissolved in 6 N HCl and hydrolyzed under nitrogen in sealed ampules for 2 h at 110°C. Hydrolysates were evaporated to dryness, redissolved in water, and phosphoamino acids detected by ascending thin layer chromatography (43). Results obtained using this chromatographic procedure were confirmed in a two-dimensional system involving high voltage electrophoresis at pH 1.9, followed by ascending chromatography (21). Samples of individual or mixed phosphoamino acids (Sigma Chemical Co., St. Louis, MO) were run in parallel lanes and detected by spraying the chromatographic plates with 3% ninhydrin dissolved in acetone. ^{32}P-labeled phosphoamino acids were located by autoradiography.

Peptide Mapping: ^{32}P-labeled sp-I components were gel purified by electroelution as described above and aliquots precipitated with acetone. Some aliquots were redissolved in 400 μl 70% formic acid, then 50 μg of sperm whale myoglobin and 400 μg of cyanogen bromide were added (1). Samples were incubated at room temperature overnight, lyophilized, and analyzed by electrophoresis on 15% polyacrylamide gels containing 0.1% SDS and 6 M urea (37). Results of a time-course experiment demonstrated that ~90% of the myoglobin molecules were cleaved at one or both of their Met residues within 1 h after the addition of cyanogen bromide. Examination of the cleavage products by gel electrophoresis revealed a pattern of completely (2.6, 6.4, and 8.2 kD) and partially (14.6 kD) cleaved peptides that remained unchanged even if cleavage was allowed to proceed for 30 h. Similarly the pattern of peptides obtained by cyanogen bromide cleavage of ^{32}P-labeled sp-I components attained a stable pattern within 1 h. Therefore, we assumed that cleavage of sp-I components was equally complete. For tryptic peptide mapping, aliquots of acetone-precipitated protein were resuspended in 50 mM ammonium bicarbonate, pH 8.0 and incubated with 200 μg trypsin overnight at 37°C. Samples were lyophilized and analyzed by two-dimensional peptide mapping on thin-layer cellulose sheets as previously described (14, 30). The first dimension involved electrophoresis at 500 V for 1.5 h in pyridine/H_2O/acetic acid (10:90:0.4). The second dimension was ascending chromatography in butanol/acetic acid/H_2O/pyridine (15:3:12:10). The second dimension was done at right angles to the first and developed twice. The fingerprints obtained from ^{32}P-labeled sp-I components remained unchanged over a 4–24-h incubation with 25–250 μg of trypsin. Thus, we conclude that trypsin cleavage was complete.

RESULTS

Identification of Secretion Polypeptide sp-Id in Extracts of Total Salivary Gland Protein

The first step required for purification of secretion polypeptides is the manual isolation of larval salivary glands. Since this is a tedious procedure, it was desirable to avoid further manipulation such as dissecting the glandular lumen (19). Thus, a procedure was sought whereby intact salivary glands could be rapidly solubilized under conditions of minimal proteolysis so that individual secretion polypeptides could be isolated from extracts of total gland protein. A variety of denaturing solvents were individually tested at concentrations up to 2.5% SDS, 8 M urea, 5 M guanidine thiocyanate, and 6 M Gdn-HCl. When solubilization of glands was observed under a dissecting microscope, each of these solutions appeared to work equally rapidly. But when these various extracts were analyzed by electrophoresis in 3–20% (wt/vol) concave exponential gradients of polyacrylamide-containing SDS, it was observed that discrete bands of low mobility, that presumably contained high M_r polypeptides, were most reproducibly obtained from extracts made with Gdn-HCl. Furthermore, Gdn-HCl extracts contained a previously undetected

secretion polypeptide.

Extracts made from salivary glands taken from 4th instar larvae typically contained three high M_r bands (e.g., see Fig. 1). Two of these bands corresponded to previously described secretion polypeptides sp-Ia and sp-Ib (12, 15, 19, 20, 30) based upon their (*a*) relative electrophoretic mobilities, (*b*) presence in fixed and microdissected salivary gland lumens, and (*c*) galactose-induced changes in steady-state glandular concentrations. The third band, however, has not been described previously. Because of its apparently high M_r and the fact that in separate experiments (data not shown) it could be isolated from the lumen of salivary glands, it was labeled sp-Id to designate it as a novel component of the sp-I family of secretion polypeptides. Biochemical similarities and differences between various sp-I components are described below.

While attempting to improve the electrophoretic resolution of sp-I components and increase the sensitivity of their detection, it was noted that alkylation of reduced sulfhydryl groups had a profound effect upon their resolution. This was best visualized by solubilizing a common pool of salivary glands in Gdn-HCl, dividing the extract in half, and adding either β-mercaptoethanol or dithiothreitol to a final concentration of 100 mM. Next, each extract was divided into aliquots to which a serial dilution of iodoacetamide was added. Each aliquot was then examined by electrophoresis on SDS gels that were stained with silver nitrate (Fig. 1). Regardless of which reductant was used, when the concentration of iodoacetamide was 5 mM or greater, alkylation differentially retarded the mobility of sp-I components (sp-Id > sp-Ib > sp-Ia) in a manner that led to enhanced separation, sharper resolution, and more sensitive detection. In converse experiments, it was shown that if either reductant was diluted sufficiently, iodoacetamide would not alter the mobility of sp-I components (data not shown). Finally, silver-stained bands corresponding to sp-Ia, sp-Ib, and sp-Id were stainable with Coomassie Brilliant Blue, sensitive to degradation by either Protease K or trypsin, and resistant to degradation by micrococcal nuclease and ribonuclease (data not shown).

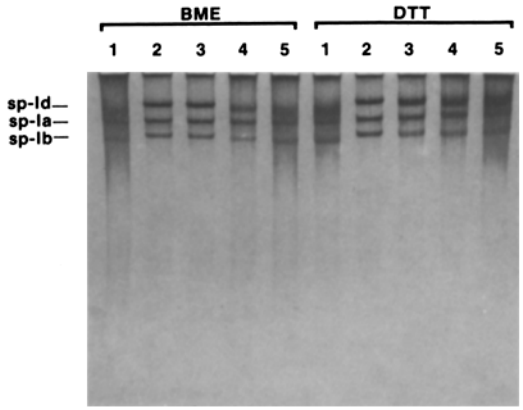

FIGURE 1 The effect of alkylation on the electrophoretic resolution of secretion polypeptides. A pool of salivary glands was solubilized in 6 M Gdn-HCl, divided in half, and either β-mercaptoethanol (*BME*) or dithiothreitol (*DTT*) added to a final concentration of 100 mM. Iodoacetamide was then added to aliquots of BME- or DTT-reduced proteins at concentrations of 0 mM (lane *1*), 100 mM (lane *2*), 25 mM (lane *3*), 5 mM (lane *4*), or 2 mM (lane *5*). Samples were alkylated, precipitated, and prepared for electrophoresis on 3–20% (wt/vol) concave exponential gradients of polyacrylamide containing SDS and stained with silver nitrate as described in Materials and Methods. Bands corresponding to secretion polypeptides (*sp-Ia*, *sp-Ib*, and *sp-Id*) are indicated to the left.

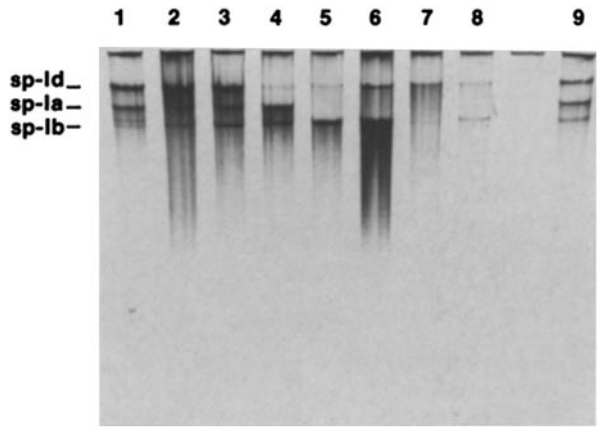

FIGURE 2 Electrophoretic pattern of secretion polypeptides obtained from salivary glands of different strains of *Chironomus*. Ten pairs of salivary glands from various strains of *C. tentans* (lanes *1–6*, and *9*) and *C. pallidivittatus* (lanes *7* and *8*) were obtained from the investigators listed below. Salivary gland protein was extracted from a single gland and examined by electrophoresis as described in Fig. 1. This gel provides a partial illustration of the diversity of patterns observed for sp-I components using samples obtained from our laboratory (lanes *1* and *9*) and the labs of B. Daneholt, Sweden (lane *2*); E. Egyhazi, Sweden (lane *3*); R. Tanguay, Canada (lane *4*); A. L. Olins, United States (*lane 5*); M. Lezzi, Switzerland (lanes *6* and *7*); and J.-E. Edstrom, West Germany (lane *8*).

The Widespread Occurrence of sp-Id

The sensitivity and resolution of the gel system just described made it routinely possible to qualitatively detect individual sp-I components in the extract of a single salivary gland. For example, to determine if the occurrence of sp-Id was restricted to our *Chironomus* stock or distributed elsewhere, a survey was conducted that spanned 11 strains of *C. tentans* and 4 strains of *C. pallidivittatus* (a closely related species). The survey used individual salivary glands supplied to us from eight European and North American laboratories. While a more detailed report of these results will be presented elsewhere (Kao, W.-Y., and S. T. Case, manuscript in preparation), one observation that was pertinent to this study was that a band with the same electrophoretic mobility as sp-Id was observed in all strains of *C. tentans* (Fig. 2, lanes *1–6* and *9*) and *C. pallidivittatus* (Fig. 2, lanes *7* and *8*) that were examined.

Identification of Phosphorylated Amino Acids in Secretion Polypeptides

Autoradiograms made from polyacrylamide gels containing salivary gland protein radiolabeled with ^{32}P-orthophosphate in vivo or in organ culture exhibited ^{32}P-labeled bands that co-migrated with sp-Ia, sp-Ib, and sp-Id (Fig. 3). Control experiments verified that these ^{32}P-labeled bands were secretion polypeptides that were resistant to nucleases and sensitive to proteases. Secretion polypeptides were then examined for their content of phosphorylated amino acids. Slices of a gel containing either ^{32}P-labeled sp-Ia, sp-Ib, or sp-Id were carefully cut out, radioactive protein was electroeluted, reprecipitated, and partially hydrolyzed in 6 N HCl at 110°C for 2 h.

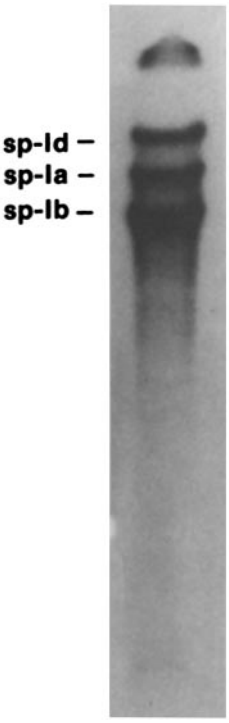

FIGURE 3 ^{32}P-labeling of secretion polypeptides. A salivary gland was removed from a 4th instar larva and incubated for 3 h in phosphate-free Cannon's medium containing ^{32}P-orthophosphate. Salivary gland protein was extracted and separated by electrophoresis as described in Fig. 1. This figure is an autoradiogram of a wet gel.

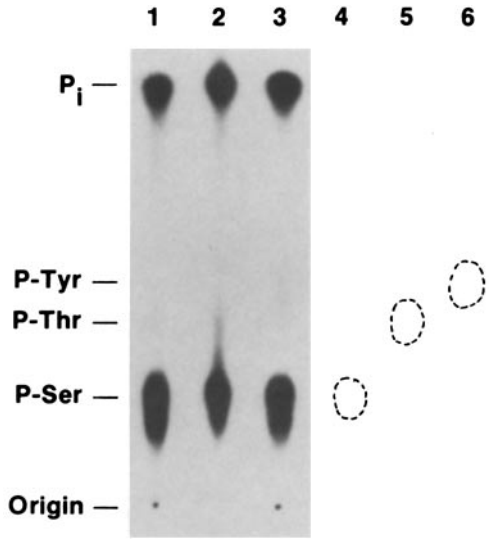

FIGURE 4 Phosphoamino acid content of secretion polypeptides. Secretion polypeptides were labeled in vivo with ^{32}P, extracted, and separated by gel electrophoresis as described for Fig. 1. Individual bands corresponding to sp-Id (lane *1*), sp-Ia (lane *2*), and sp-Ib (lane *3*) were localized by autoradiography, electroeluted, precipitated, and hydrolyzed in 6 N HCl at 110°C for 3 h. Each hydrolysate was then fractionated by thin-layer ascending chromatography in parallel with samples of authentic P-Ser (lane *4*), P-Thr (lane *5*), and P-Tyr (lane *6*). Phosphoamino acid standards were located by staining with ninhydrin, whereas radioactive phosphoamino acids were located by autoradiography.

Hydrolysates were analyzed by thin layer chromatography in parallel with authentic phosphoamino acids. Autoradiograms demonstrated that most of the ^{32}P incorporated into sp-Ia, sp-Ib, and sp-Id co-migrated with P-Ser (Fig. 4). Similar results were obtained when hydrolysates were analyzed by two-dimensional separations (21).

Cyanogen Bromide and Trypsin Cleavage Patterns of Secretion Polypeptides

Gel-purified samples of ^{32}P-labeled sp-Ia, sp-Ib, and sp-Id were compared for structural similarities and differences by peptide mapping under conditions of apparently complete cleavage (see Materials and Methods). For example, each radiolabeled sp-I component was mixed with sperm whale myoglobin, incubated overnight with an excess of cyanogen bromide, and analyzed by one-dimensional electrophoresis on 15% polyacrylamide gels. Myoglobin peptides and parallel M_r markers were located by staining the gels, and ^{32}P-labeled sp-I peptides were located by autoradiography (Fig. 5). All three ^{32}P-labeled sp-I components were cleaved into a simple ladder-like pattern: a major intense band of ~M_r 11,000–14,000 and a subset of one or more minor bands of decreasing intensity and increasing M_r. The number of minor bands sometimes varied and their visualization could be enhanced by prolonged autoradiography (Fig. 5, lane *3*). The apparent M_r of these minor bands were approximately integral multiples (1×, 2×, 3×, etc.) of the M_r of their respective major bands. Under conditions of partial cleavage (data not shown), the relative intensity of each major band decreased, while the number of high M_r minor bands increased. A peptide (M_r ~ 17,000) of variable intensity (Fig. 5, lanes *2* and *3*) was the most frequent example of a band that did not fit the ladder. Overall, sp-I components could not be distinguished from one another by cleavage with cyanogen bromide.

Two-dimensional fingerprints of ^{32}P-labeled sp-I tryptic peptides were also constructed. Tryptic fingerprints have been repeated six times over a 10-fold concentration range of enzyme on different preparations of individual secretion polypeptides. Autoradiograms from replicate experiments were compared to each other, and a composite diagram was made for each sp-I component. In addition, autoradiograms from different sp-I components were compared to one another. Spots were grouped according to their electrophoretic properties (*A*, acidic; *B*, neutral; *C*, basic) and those that were clearly observed to have a unique identity were numbered. The autoradiograms shown in Fig. 6 were from one experiment using a single preparation of sp-I components, whereas the artistic interpretations shown below the autoradiograms represent the composite data for apparently complete cleavage of each sp-I component.

Unlike the cyanogen bromide cleavage patterns that provided little or no distinction between sp-I components, qualitative and apparently quantitative differences were observed in the fingerprint of phosphorylated sp-I tryptic peptides (Fig. 6). To begin with, sp-Id had the simplest pattern of phosphorylated tryptic peptides characterized by four intense spots: A2, A4, B2, and C1 (Fig. 6*A*). By comparison, sp-Ia had the most complex pattern with at least seven major spots: A1, A2, A3, A4, B3, B4, and C1 (Fig. 6*B*). Finally, sp-Ib had nearly as many spots as did sp-Ia but most of the autoradiographic intensity was confined to A6, B4, and C1 (Fig. 6*C*). Some distinctive features of each sp-I component can be summarized as follows: B2 was only observed in sp-Id which lacked A1 and A3; sp-Ia had two pairs of equally intense major spots (A1/A2 and A3/A4); sp-Ib also contained the pairs A1/A2 and A3/A4 but their intensities were consistently less than that of A6 which appeared to be a characteristic spot for this polypeptide.

The striped area near the bottom of each panel represents

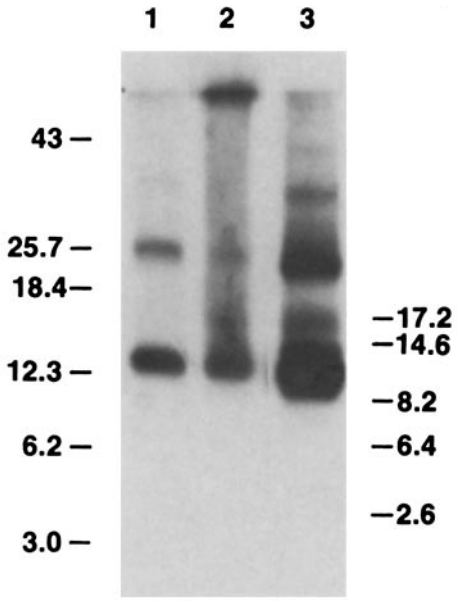

FIGURE 5 Cyanogen bromide cleavage of secretion polypeptides. ^{32}P-labeled samples of sp-Id (lane *1*), sp-Ia (lane *2*), and sp-Ib (lane *3*) were electroeluted from gels as described in Fig. 4, precipitated, and redissolved in 70% formic acid. Before the addition of cyanogen bromide in excess, 50 μg of sperm whale myoglobin was added to each sample to provide internal references for both the cleavage reaction and M_r markers. Samples were separated on 15% polyacrylamide gels containing SDS. M_r markers were located by staining the gel with Coomassie Brilliant Blue, and ^{32}P-labeled peptides were located by autoradiography. Numbers to the left indicate the size ($M_r \times 10^{-3}$) of markers in parallel lanes (*43*, ovalbumin; *25.7*, α-chymotrypsinogen; *18.4*, β-lactoglobulin; *12.3*, cytochrome *c*; *6.2*, bovine trypsin inhibitor; *3.0*, insulin). Numbers to the right indicate the size ($M_r \times 10^{-3}$) of intact (*17.2*), partially (*14.6*), or completely (*8.2*, *6.4*, and *2.6*) cleaved fragments of myoglobin. Lane *3* was overexposed to enhance visualization of less intense high M_r bands.

material that was not resolved in this solvent system. The autoradiographic intensity of this area remained proportional to the radioactivity applied to the chromatogram and it was not reduced by further addition of trypsin. Although we have not characterized this material, its chromatographic behavior is similar to that of glycopeptides (27).

Galactose-induced Alterations in the Electrophoretic Display of Secretion Polypeptides

Edstrom et al. (12) first reported that addition of galactose to cultures of *C. pallidivittatus* larvae results in a correlation between the electrophoretic display of individual secretion polypeptides (sp-Ia, sp-Ib, and sp-Ic) that directly coincides with changes in the occurrence of puffs at chromosomal loci that form BRs (BR1, BR2, and BR6, respectively; reference 4). Since Beermann (4) also reported that identical cytological changes take place in *C. tentans*, we wanted to determine if a similar correlation existed in the appearance of sp-I components. More importantly, we wanted to determine what effect, if any, galactose had on the synthesis of sp-Id.

Sibling larvae were divided into two cultures that contained either normal laboratory culture medium (0.04% NaCl) or culture medium plus 0.5% galactose. At various time intervals, larvae were taken from both cultures and their paired salivary glands removed. One gland was used for a cytological examination of puffing patterns, while its sister gland was examined for its content of sp-I components. In some instances, salivary glands were labeled with [^{35}S]Met to radiolabel polypeptides that were synthesized immediately before extraction of salivary gland protein.

The steady-state level (silver-stained) versus newly synthesized (^{35}S-labeled) patterns of sp-I components obtained from individual larvae were compared over a series of time course experiments. Fig. 7 illustrates a crucial observation that was

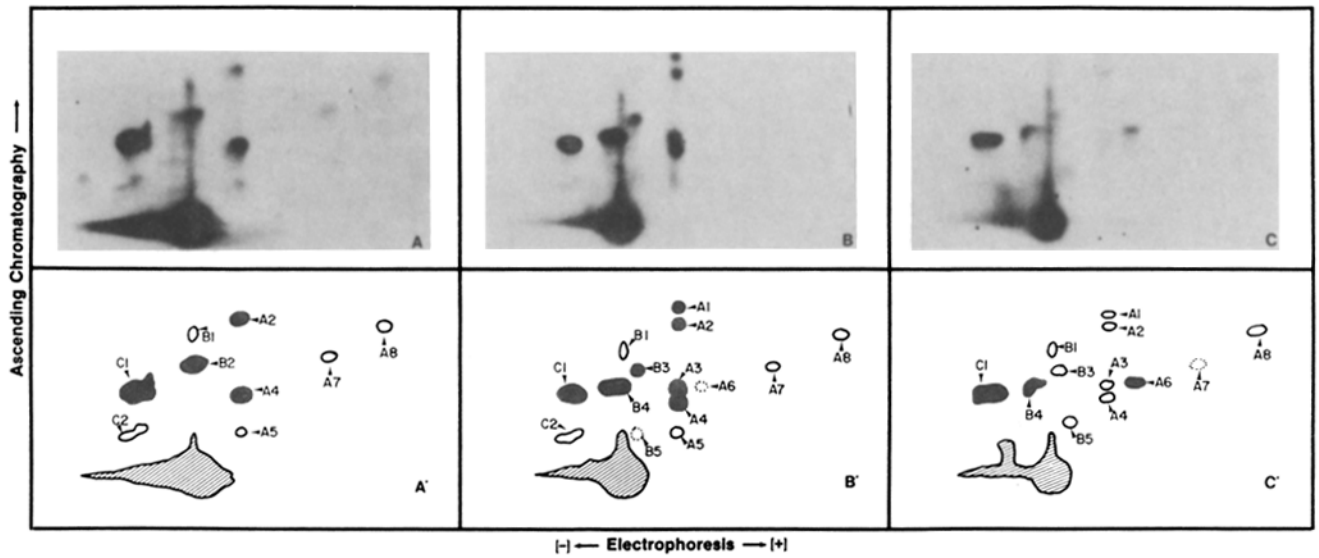

FIGURE 6 Two-dimensional tryptic fingerprints of secretion polypeptides. ^{32}P-labeled samples of sp-Id (*A*), sp-Ia (*B*), and sp-Ib (*C*) were obtained from gels as described in Fig. 4. Each sample was incubated with an excess of trypsin and fractionated on thin-layer cellulose sheets by electrophoresis in pyridine/acetic acid/H_2O (10:0.4:90) (cathode to the left, anode to the right) followed by ascending chromatography in butanol/acetic acid/H_2O/pyridine (15:3:12:10). The upper panels show a parallel set of autoradiograms from one experiment. The lower panels show artistic interpretations of autoradiograms summarized over a series of six experiments. The black spots were reproducibly most intense, white spots were reproducible but comparatively less intense, and dotted spots were not reproducible. The origin was in the central portion of the striped area, which represents material that was not resolved.

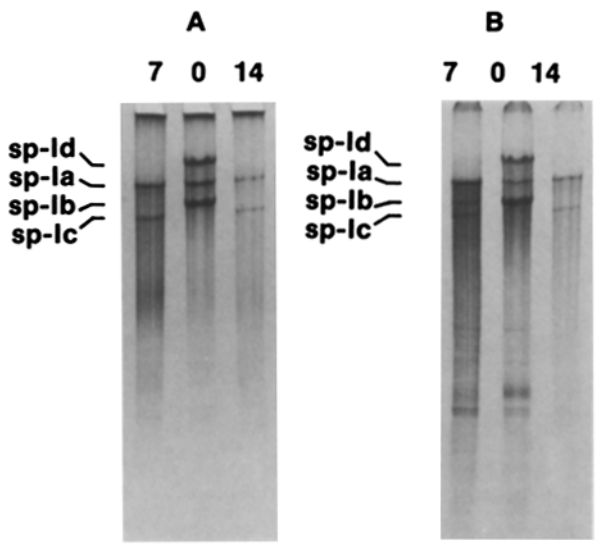

FIGURE 7 Galactose-induced alterations in the electrophoretic pattern of secretion polypeptides. Salivary glands were removed from sibling larvae that were exposed to 0.5% galactose for 0, 7, and 14 d. Glands were incubated in Cannon's medium containing [^{35}S]Met for 3 h just before extracting salivary gland protein in Gdn-HCl. Samples were reduced, alkylated, and stored at −20°C in acetone until all were available for electrophoresis. After electrophoresis, the gel was stained with silver nitrate (*A*), dried, and autoradiographed (*B*). Each lane contains the extract from a single gland.

made: a sequential alteration took place in the synthesis of three out of four sp-I components. sp-Ia was detected as a silver-stained and ^{35}S-labeled band throughout the duration of the experiment, although there was often a decrease in total protein recovered from larvae exposed to galactose for prolonged periods of time. By comparison, the level of sp-Ib gradually declines in response to galactose. At 7 d, sp-Ib was barely visible by staining yet it still labeled with ^{35}S. After 14 d, sp-Ib was not detectable by either method. sp-Ic was not detected as a stained or radiolabeled band in control larvae. However, it was observed by 7 d and persisted so long as the exposure to galactose continued. Finally, the level of sp-Id also declined in response to galactose but reached nondetectable levels before sp-Ib. The stained and ^{35}S-labeled patterns of sp-I components in control larvae remained unchanged throughout the duration of this experiment.

Results of the cytological examination of galactose-induced alterations in puffing at BR loci (data not shown) were in complete accord with the observations of Beermann (4): the puff at BR1 either remained unchanged or increased in size; the puff at BR2 completely regressed concurrent with the induction of a new puff at BR6.

DISCUSSION

Detection and Resolution of Giant Secretion Polypeptides

Within this paper, we presented a simple and efficient procedure for solubilization of *Chironomus* salivary glands and subsequent detection of apparently undegraded high M_r secretion polypeptides. The enhanced resolution obtained by a combination of our extraction and electrophoresis protocols resulted in the detection of sp-Id, a novel giant secretion polypeptide. We demonstrated that sp-Id was a polypeptide by comparing it to sp-Ia and sp-Ib in (*a*) staining with Coomassie dye or silver nitrate (Fig. 1), (*b*) sensitivity to proteolytic enzymes and resistance to nucleases (data not shown), and (*c*) labeling with ^{35}S[Met] (Fig. 7). In addition, we have observed that all three of these sp-I components stain with periodic acid Schiff (data not shown) suggesting that, similar to sp-Ia and sp-Ib (20), sp-Id is probably glycosylated.

The level of detection and resolution achieved in this study made it possible to examine the secretion polypeptide content of individual salivary glands which average 1–2-mm long. We applied this capability to an intra- and interspecific survey of more than a dozen strains of *C. tentans* and *C. pallidivittatus*. It should be noted that primary identification of individual sp-I components in such a survey is currently limited to their electrophoretic comigration on SDS gels. In conjunction with this, however, we were able to test some strains before and after their exposure to galactose and confirm that there was a parallel increase or decrease in the synthesis of electrophoretic equivalents (i.e., results similar to those in Fig. 7). With the above limitation in mind, we concluded that the electrophoretic equivalent of sp-Id was widespread in all strains of *Chironomus* that were examined (Fig. 2).

sp-Id May Be Encoded by Another BR-like Gene

Analyses of cloned BR DNA in *C. tentans* and *C. pallidivittatus* indicate that BR1 (6, 11, 42), BR2 (5, 22, 36, 41), and BR6 (15, 26) genes are comprised of 100 or more tandemly repeated copies of 180–246-bp protein coding sequences. Each basic repeat is divided approximately in half into a constant (C) region and a subrepeat (SR) region containing shorter tandem repeats of variable length and number. BR mRNAs contain such repeated sequences (24, 40) and can be translated in vitro into giant secretion polypeptides (31, 39). However, the size of BR mRNAs and in vivo posttranslational modification of sp-I components have made it technically impossible to directly demonstrate which BR gene encodes each sp-I component (e.g., using in vitro translation of hybrid-selected clone-purified BR mRNA). To date the best available data for gene/polypeptide assignments is based upon direct correlations in BR puffing and the detection of sp-I components (12): sp-Ia is presumably encoded by BR1α repeats; sp-Ib may be encoded by a gene comprised of BR2α and BR2β repeats; and sp-Ic is most likely encoded by BR6α repeats.

Several biochemical properties of sp-I components that are presented here were compatible with predictions that can be made if one assumes that most of an sp-I polypeptide is comprised of tandemly repeated amino acid sequences encoded in BR DNA. For example, one would predict that Cys residues were present for reduction and alkylation to modify the electrophoretic mobility of sp-Ia, sp-Ib, and sp-Id (Fig. 1). All four BR repeats encode four Cys residues at conserved positions within C regions (Fig. 8). Since there may be as many as 100 repeats per BR mRNA molecule, there could be as many as 400 Cys residues per sp-I polypeptide.

sp-Ia, sp-Ib, and sp-Id were shown to be phosphorylated almost exclusively at Ser residues (Figs. 3 and 4), whereas sp-Ic is not phosphorylated (15). At least 11 Ser residues are encoded throughout C and SR regions of BR1α, BR2α, and BR2β repeats. However, BR6 repeats do not code for Ser, Thr, or Tyr (Fig. 8).

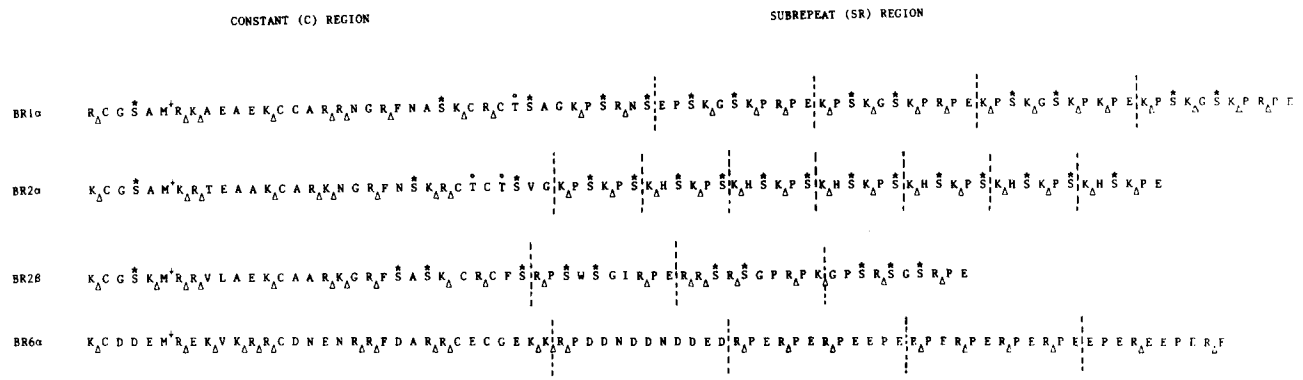

FIGURE 8 Polypeptides encoded in cloned BR DNA. Amino acid sequences were derived from BR1α (6, 42), BR2α (36), BR2β (8, 41), and BR6α (26) repeat units cloned from *C. tentans*. Constant (C) and subrepeat (*SR*) regions are shown, and each subrepeat is demarcated by a vertical dotted line. Potential sites for phosphorylation ($\overset{*}{S}$, $\overset{*}{T}$), cyanogen bromide (↓), and trypsin (Δ) cleavage are shown.

All sp-I components incorporated ^{35}S[Met] (Fig. 7), and cyanogen bromide cleavage of ^{32}P-labeled sp-Ia, sp-Ib, and sp-Id (Fig. 5) yielded peptides of M_r 11,000–13,000. Because our autoradiograms for each of these sp-I components were similar to stained gels containing an unfractionated mixture of sp-I components cleaved with cyanogen bromide (32), we concluded that Met residues are spaced at regular intervals in sp-Ia, sp-Ib, and sp-Id. In fact, each BR repeat encodes a single Met residue at a conserved position within C regions. There is, however, a discrepancy in that the predicted molecular weight of cyanogen bromide peptides encoded by cloned BR DNAs would have an M_r of 7,000–9,000. sp-I components are glycosylated (20) and phosphorylated (reference 15; Fig. 3), and potential sites for these modifications occur within each repeat (see Fig. 8). It therefore seems reasonable to conclude that the apparent M_r of sp-I cyanogen bromide peptides was overestimated because posttranslational modifications caused a decrease in their electrophoretic mobility relative to M_r markers.

Finally, trypsin cleavage of ^{32}P-labeled sp-Ia, sp-Ib, and sp-Id yielded two-dimensional peptide maps that were simple, considering that such large proteins could potentially generate rather complex patterns. Since apparently complete cleavage of each sp-I component reproducibly generated major and minor spots of autoradiographic intensity (Fig. 6), we concluded that some tryptic peptides contained more ^{32}P molecules than others and/or all tryptic peptides do not occur in an equimolar ratio. One can also conclude that sp-I components were structurally similar because they exhibited an overlapping pattern of peptides, yet they are not identical because each map had some unique characteristic. Although we cannot rule out the possibility that subtle differences we observed between the fingerprints of sp-I components were simply due to a few posttranslational modifications, all of the above conclusions are also consistent with predictions that can be made from amino acid substitutions that occur in BR DNA (Fig. 8). There were insufficient quantities of material for us to perform peptide isolation and compositional analysis to distinguish between these alternatives directly.

In summary, the observed number of similarities and subtle differences in the apparent M_r, phosphorylation, and peptide maps of sp-I components led us to conclude that sp-Id is translated from mRNA whose size and organization of repeated nucleotide sequences is remarkably similar to the BR mRNAs that encode sp-Ia, sp-Ib, and sp-Ic.

Where Is the Gene for sp-Id?

We will presently omit any consideration of alternative posttranscriptional processing because BR mRNAs are not known to undergo splicing (7, 10). Instead, we would like to consider the simple notion that sp-Id is encoded by a gene that is distinct from the genes that encode sp-Ia, sp-Ib, and sp-Ic and speculate about its identity and location.

A fundamental concept in this biological system has been that secretion polypeptides are encoded by genes located in salivary gland–specific BRs (3, 17). By analogy to other sp-I components and their presumptive genes, the gene for sp-Id should be found at a locus which, during the 4th instar of larval development (*a*) routinely puffs on salivary gland polytene chromosomes of *C. tentans* and *C. pallidivittatus* (4, 17), (*b*) displays intense transcription autoradiograms (29, 32), (*c*) contains a high concentration of RNA polymerase II (32), and (*d*) has characteristic 500-nm ribonucleoprotein particles known as BR granules that contain BR 75S RNA (34, 35). The only loci that meet all of these criteria are BR1 and BR2. Is it possible that one of these puffs contains two genes for secretion polypeptides?

We demonstrated that galactose repressed the synthesis of both sp-Ib and sp-Id (Fig. 7). Consequently, it seems reasonable to expect the BRs that contain each gene to regress. The puff at BR1 can arise independently from band IV-2-A7 or IV-2-A11 (2). Though it is plausible that the puff from one of these bands is sensitive to galactose and the other is not, such an idea would be inconsistent with the observed expansion of BR1 (reference 4; our unpublished data) and increased BR1 RNA synthesis (28).

On the other hand, BR2 is the only puff that regresses in response to galactose (4, 12), concurrent with cessation of RNA synthesis at the BR2 locus (28). Furthermore, BR2 is the only puff from which two distinctly different (BR2α and BR2β) repeats have been cloned. Even though BR2α and BR2β repeats were never isolated within a single clone, a variety of indirect data implied that BR2α and BR2β repeats exist as two contiguous blocks within a single BR2 gene (5, 8, 41). However, all previous observations plus data presented in this report would be more compatible with a model whereby BR2α and BR2β repeats are contained within two separate BR2 genes. Further experiments are aimed at directly testing this hypothesis.

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