Turnover and Orientation of the Major Neural Retina Cell Surface Protein Protected from Tryptic Cleavage by Calcium†

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ABSTRACT: Calcium protects a limited number of embryonic chick neural retina cell surface proteins from tryptic cleavage. One glycoprotein of M_r $\sim 1.3 \times 10^5$ and pI $\sim$ 4.8 (gp130) is present in intact retinas and, in the presence of Ca^{2+}, is both resistant to tryptic cleavage and poorly iodinated. When cultured in vitro, iodinated retinas release into the medium a number of iodinated polypeptides; one of the major iodinated components is a polypeptide of M_r $\sim 9 \times 10^4$ and pI $\sim$ 4.8 (gp90). This component is also resistant to tryptic cleavage in the presence of Ca^{2+}. Two-dimensional peptide maps of gp130 and gp90 derived respectively from iodinated retinas and their conditioned media are very similar. Maps of samples reiodinated following denaturation show the same similarities as well as additional labeled peptides. Furthermore, the two-dimensional peptide maps of the two molecules prepared from retinas cultured in the presence of [^{3}H]glucosamine are identical. We conclude that gp90 is a turnover fragment of gp130 and comprises that portion of gp130 exposed at the cell surface. The relevance of these polypeptides to Ca^{2+}-dependent retina cell–cell adhesion and their similarity to polypeptides implicated in Ca^{2+}-dependent adhesion of other cell types are discussed.

The effects of including Ca^{2+} during trypsinization of embryonic chick neural retinas have been examined in detail by our laboratory. One aspect of this work has been the role of Ca^{2+} in protecting a limited number of cell surface proteins from tryptic cleavage (Cook & Lilien, 1982). A cell surface glycoprotein of M_r $\sim 1.3 \times 10^5$ and pI $\sim$ 4.8 (gp130) is of particular interest; it is present in intact retinas and, in the presence of Ca^{2+}, is both resistant to tryptic cleavage and poorly iodinated. However, labeling of gp130 on intact cells by treatment with periodate or galactose oxidase and subsequent reduction with borotritide is unaffected by Ca^{2+}, indicating that certain oligosaccharide moieties of gp130 remain exposed at the cell surface in the presence of Ca^{2+}.

The behavior of cell populations is also affected by the inclusion of Ca^{2+} during trypsinization. Unlike cells prepared by standard procedures, those prepared by trypsinization in the presence of Ca^{2+} have a functionally intact Ca^{2+}-dependent adhesive system (Takeichi et al., 1979; Grunwald et al., 1980; Magnani et al., 1981; Brackenbury et al., 1981). Antibodies raised to cells prepared in this way immunoprecipitate gp130, and their Fab′ fragments inhibit Ca^{2+}-dependent intercellular adhesion (Grunwald et al., 1982). Both the immunoprecipitation of gp130 and the inhibition of adhesion are blocked by a fraction of retina conditioned medium. A protein of M_r $\sim 9 \times 10^4$ and pI $\sim$ 4.8 is highly enriched in this fraction and is the major constituent immunoprecipitated by the above antibodies. These data led us to postulate that the M_r $\sim 9 \times 10^4$ species was a fragment of gp130.

In this paper, we demonstrate that the M_r $\sim 9 \times 10^4$ protein is glycosylated (gp90, therefore) and is one of the major labeled components which accumulates in the medium during the culture of iodinated retinas. Further, we show that, like gp130 at the cell surface, soluble gp90 is resistant to tryptic cleavage in the presence of Ca^{2+}. Lastly, we find many similarities in the two-dimensional tryptic peptide maps of gp130 and gp90. These data support our hypothesis that gp130 is the precursor of gp90. The relevance of these findings to Ca^{2+}-dependent intercellular adhesion is also discussed.

Materials and Methods

Materials. Reacti-gel 6X and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen) were purchased from Pierce Chemical Co. (Rockford, IL). Acrylamide (electrophoresis grade), *N*,*N*′-methylenebis(acrylamide) (electrophoresis grade), sodium dodecyl sulfate (SDS; electrophoresis grade), and cellophane sheets were obtained from Bio-Rad Laboratories (Richmond, CA). Urea (ultrapure) was supplied by Schwarz/Mann Division, Becton, Dickinson & Co. (Orangeberg, NY). AG 2-11 Servalytes were obtained from Accurate Chemical and Scientific Corp. (Hicksville, NY). Antipain, leupeptin, chymostatin, phenylmethanesulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane (Tris), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), methyl α-mannoside, 1-butanol, and diphenylcarbamoyl chloride treated trypsin (DPCC–trypsin) were purchased from Sigma Chemical Co. (St. Louis, MO). Formic acid and pyridine were supplied by Aldrich Chemical Co. (Milwaukee, WI). Plasma albumin (bovine) was obtained from Reheis Chemical Co. (Chicago, IL). Prestained molecular weight markers (high range) and Nonidet P-40 (NP-40) were purchased from Bethesda Research Labs., Inc. (Gaithersburg, MD). Concanavalin A (Con A) and deoxyribonuclease (DNase, bovine pancreas) were obtained from Calbiochem-Behring Corp. (San Diego, CA). Contaminating proteolytic activity in DNase was abolished by treatment with PMSF as previously reported (Grunwald et al., 1980). Carrier-free Na^{125}I (iodination grade) was purchased from New England Nuclear (Boston, MA). X-ray film (XAR-5) was supplied by Eastman Kodak Co. (Rochester, NY). Cronex Lightning-Plus intensifying screens were obtained from E. I. du Pont de Nemours & Co. (Wilmington, DE).

Iodination of Retinas. Neural retinas were dissected from 9–10-day-old white Leghorn chick embryos and iodinated by the Iodogen method (Fraker & Speck, 1978) as described previously (Cook & Lilien, 1982). Briefly, the retinas were washed free of Ca^{2+}, incubated at 37 °C in Ca^{2+}-free buffer containing 2 mM EGTA, and then iodinated at 4 °C in the

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absence of Ca^{2+}. After iodination, the retinas were exhaustively washed to remove the bulk of the free $Na^{125}I$.

Preparation of Medium Conditioned by Iodinated Retinas. Iodinated retinas were cultured in Hepes (10 mM, pH 7.0) buffered saline (0.14 M) containing glucose (10 mM), KCl (5 mM), $MgCl_2$ (1 mM), and $CaCl_2$ (1 mM) at one retina per mL for 8–12 h at 37 °C on a rotary shaker at 40 rpm. The conditioned medium was centrifuged (10^4g, 20 min, 4 °C) and either precipitated at −20 °C with EtOH (5 volumes) after the addition of bovine serum albumin (BSA) (0.1 mg/mL) as carrier or processed, as described below, for tryptic digestion.

Tryptic Digestion of Medium Conditioned by Iodinated Retinas. Medium conditioned for 8 h at 37 °C by iodinated retinas was precipitated with EtOH (0.7 volume) at −20 °C after the addition of $MgCl_2$ (5 mM) and sodium phosphate (6 mM, pH 7.4). The precipitate was recovered by centrifugation (10^4g, 20 min, 4 °C) and washed 3 times by resuspension and centrifugation, twice with EtOH (1 volume, −20 °C) and once with acetone (1 volume, −20 °C). The final pellet was air-dried and then taken up in a solution of EDTA (0.1 M, pH 6.0, ~0.1 volume). The material was then exhaustively dialyzed (4 °C) first against the above EDTA solution and finally against several changes of Hepes (50 mM, pH 7.4) buffered saline (0.15 M). After centrifugation (10^4g, 20 min, 4 °C) the supernatant was held at −20 °C until use.

Divalent cation free samples of concentrated conditioned medium were supplemented with the indicated cation (Ca^{2+}, Mn^{2+}, or Mg^{2+}; all as chloride salts) at the concentration noted (0–10 mM), incubated for 15 min at 37 °C, and then digested with trypsin (100 μg/mL) for 15 min at 37 °C. After the reaction was terminated with PMSF (10 mM), the samples were held at −20 °C until analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or two-dimensional gel electrophoresis.

Processing of Iodinated Retinas. Iodinated retinas were homogenized in ice-cold Hepes buffer (10 mM, pH 7.5) containing $MgCl_2$ (1 mM), DNase (100 μg/mL), antipain (50 μg/mL), and chymostatin (50 μg/mL) at five retinas per mL by repeated passage (50 times) through a 22-gauge needle. The homogenate was centrifuged [$(2 \times 10^4)g$, 30 min, 4 °C] and the pellet washed with the same buffer by homogenization and centrifugation as above. This material was used as the source of gp130 for peptide mapping without reiodination. For peptide mapping after reiodination, the pellet was extracted twice as described above with the same buffer supplemented with NP-40 (1%) and NaCl (0.15 M). The pellets were held at −70 °C until two-dimensional gel electrophoresis.

Partial Purification of gp90 and Iodination in Solution. Retina-conditioned medium was prepared, concentrated, and fractionated by gel filtration as previously described (Grunwald et al., 1982) except that the gel filtration was performed in Hepes (50 mM, pH 6.8) buffered saline (0.15 M) containing $CaCl_2$ (1 mM) and NaN_3 (5 mM). After the addition of antipain (50 μg/mL), chymostatin (50 μg/mL), and EGTA (2 mM), pooled fractions (ca. 200 μg/mL protein) containing gp90 were iodinated by the Iodogen method (Fraker & Speck, 1978); 5 mCi/mL (ca. 2 μM) $Na^{125}I$ and 5 μg of Iodogen/mL of solution were used, and the reaction was carried out for 10 min on ice. The reaction was terminated by removal of the sample from the iodination tube and the addition of tyrosine (500 μM). Prior to Con A binding, $CaCl_2$ (3 mM) and $MnCl_2$ (1 mM) were added, and the sample was held for 30 min on ice before adding NP-40 (1%) and BSA (1 mg/mL). The sample was then centrifuged [$(2 \times 10^4)g$, 30 min, 4 °C] and the supernatant incubated for 1 h at 4 °C with Con A–agarose (0.1 volume) which had been washed with Hepes (10 mM, pH 6.5) buffered saline (0.15 M) containing $CaCl_2$ (1 mM), $MnCl_2$ (1 mM), NP-40 (1%), antipain (50 μg/mL), chymostatin (50 μg/mL), and BSA (1 mg/mL). Unbound material was removed by extensive washing with the above buffer. After a wash with the above buffer lacking $CaCl_2$ and $MnCl_2$, bound material was eluted with ethanolamine (50 mM, pH 11.5) buffered methyl α-mannoside (0.5 M) containing NP-40 (1%), antipain (50 μg/mL), chymostatin (50 μg/mL), and BSA (1 mg/mL). Elution was repeated and the pooled eluate neutralized by the addition of Hepes (100 mM, final pH ~7.5). The eluate was then centrifuged [$(2 \times 10^4)g$, 30 min, 4 °C] and the supernatant precipitated with EtOH (5 volumes) at −20 °C. The precipitate was recovered by centrifugation [$(2 \times 10^4)g$, 30 min, 4 °C] before two-dimensional gel electrophoresis.

Preparation of Con A–Agarose. Concanavalin A was coupled to the highly reactive imidazolyl carbamate derivative of beaded agarose (Reacti-gel 6X). Reacti-gel 6X was hydrated as per the manufacturer's instructions and was resuspended (1:1) in 1 M Na_2CO_3 (pH 10) containing concanavalin A (20 mg/mL) and 0.5 M methyl α-mannoside. The suspension was agitated on a shaker for 48 h at 4 °C. After this reaction time, the gel was washed 3 times with 2 volumes of 0.5 M NaCl buffered alternatively with 0.1 M sodium acetate (pH 4) and 0.1 M $NaHCO_3$ (pH 8.3). The gel was finally washed with 5 volumes of Hepes (10 mM, pH 6.8), buffered saline (0.15 M) and stored as a 1:1 suspension in this buffer containing 0.5 M methyl α-mannoside. Typical preparations contained between 4.9 and 7.5 mg of concanavalin A/mL of packed agarose gel.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE) was performed in slabs by using the discontinuous buffer system of Laemmli (1970). A 3% stacking gel and either uniform 7.5% or 5–10% gradient separating gels were used [30:1 acrylamide–methylenebis(acrylamide)]. Samples were taken up in Tris-HCl buffer (62.5 mM, pH 6.8) containing SDS (3%), 2-mercaptoethanol (5%), glycerol (10%), and bromphenol blue (50 μg/mL) and heated (100 °C, 5 min) prior to loading.

Isoelectric focusing (IEF) was carried out as described by O'Farrell (1975) with several modifications, most of which have been reported (Cook & Lilien, 1982). In this work, the IEF gels were prefocused more extensively (30 min at 200 V and 30 min at 400 V), the IEF sample buffer lacked PMSF and contained chymostatin (50 μg/mL), and the IEF gels were equilibrated for 30 min each in three to four changes (5 mL) of Tris-HCl buffer (62.5 mM, pH 6.8) containing SDS (3%), 2-mercaptoethanol (5%), and bromphenol blue (50 μg/mL). The above SDS–PAGE system was used as the second dimension.

Analytical slabs were stained and dried as previously described (Cook & Lilien, 1982) and autoradiographed (Kodak XAR-5) at −70 °C with intensifying screens (Du Pont Cronex Lightning-Plus). When the recovery of proteins for peptide mapping or reiodination was desired, slabs were dried without fixation or staining. They were equilibrated (1 h, 25 °C) in aqueous glycerol (200 mL, 20%) and dried under heat (80 °C) and vacuum between sheets of cellophane which had been boiled in a solution of sodium carbonate (5%), SDS (1%), and EDTA (50 mM) and thoroughly rinsed with distilled water. The dried slabs were marked with $Na^{125}I$ containing ink and then autoradiographed as described above. The slabs were held under vacuum when autoradiography or excision of spots

(see below) was delayed for more than a few hours.

After the slab was aligned with its autoradiograph, the desired spot was outlined with a pen and excised. Extreme care was taken to minimize contamination. The excised spots were stored under vacuum at −20 °C.

Reiodination of Proteins after Two-Dimensional Gel Electrophoresis. Our protocol is derived from those of Elder et al. (1977) and Bryant et al. (1979). Reiodination was performed by the Iodogen method (Fraker & Speck, 1978) in Tris-HCl buffer (125 mM, pH 6.8) containing SDS (0.1%) and $Na^{125}I$ (5 mCi/mL, ca. 2 μM). This solution (250 μL) was transferred to a glass tube containing Iodogen (13 μg) and the dry spot added. After 1 h at 25 °C on a rotary mixer, the spot was transferred to a fresh tube and equilibrated at 25 °C for 30 min each with two aliquots (500 μL) of Tris-HCl buffer (62.5 mM, pH 6.8) containing SDS (3%), 2-mercaptoethanol (5%), and glycerol (10%). The spots were heated for 5 min at 90–100 °C, and the proteins were recovered by SDS–PAGE (7.5% slabs). In some experiments, the reiodinated spots were further equilibrated twice as described with the above buffer containing iodoacetamide (0.1 M) in place of 2-mercaptoethanol; the recoveries, especially of gp90, were lower, but the peptide maps seemed to have lower backgrounds. The slabs were dried and autoradiographed and the protein bands excised as described above. The excised bands were held under vacuum at −20 °C.

Tryptic Peptide Mapping. The procedure used is based on those of Elder et al. (1977) and Bryant et al. (1979). All glassware was cleaned with aqua regia before use. The piece of dry polyacrylamide was equilibrated for 30 min each in three aliquots (1 mL) of ammonium bicarbonate buffer (50 mM, pH 8.0) containing chymostatin (25 μg/mL) and then cut into 1–2-mm^3 fragments. Tryptic digestion was performed in the chymostatin-containing bicarbonate buffer (500 μL) for 24 h at 37 °C; DPCC–trypsin (50 μg/mL) was added both at the start and after 8 h of digestion. The supernatant was removed, and the samples were incubated with fresh digestion buffer (500 μL) for 6 h at 37 °C. After being filtered through glass wool, the pooled supernatants were concentrated by lyophilization. Between 90% and 95% of the label in the sample was routinely eluted. The peptides were held under vacuum at −20 °C until mapping.

The tryptic peptides were electrophoresed on paper (Whatman 3MM) in formic acid (30%) for 20 min at 50 V/cm (20-cm length). After drying (2 h at 60 °C), each lane was cut out and sewed to a sheet (20 cm by 30 cm) of 3MM paper. The papers were equilibrated (3 h) in the tank and chromatographed in the ascending mode with acetic acid–pyridine–butanol–water (3:10:15:12) until the solvent front had moved ca. 24 cm (ca. 7 h). The chromatograms were dried (3 h at 60 °C) and then autoradiographed (Kodak XAR-5) at −70 °C with intensifying screens (Du Pont Cronex Lighting-Plus).

Results and Analysis

Labeled gp90 Accumulates in the Medium during Culture of Iodinated Embryonic Retinas. Cell surface proteins are preferentially labeled during the iodination of intact embryonic retinas (Cook & Lilien, 1982). Iodination in the absence of Ca^{2+} labels a limited number of proteins; gp130 is one of the most heavily labeled (Figure 1a, arrowhead). During in vitro culture of such iodinated retinas, many labeled proteins accumulate in the medium (Figure 1b); gp90 (indicated by arrowhead) is one of the major iodinated species. Since gp90 is a major labeled protein in the conditioned medium and is not appreciably labeled in iodinated retinas before culture, it must be derived from one of the more heavily labeled proteins in iodinated retinas.

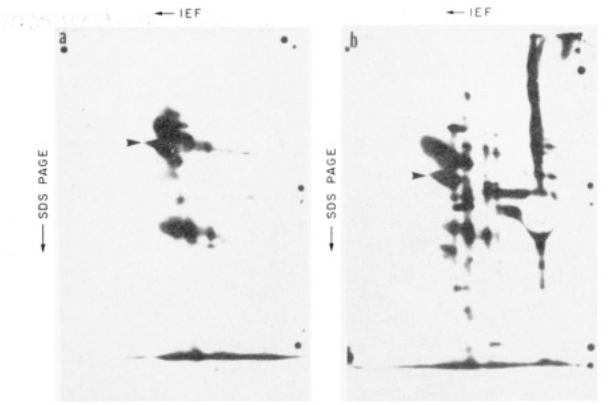

FIGURE 1: Two-dimensional gels of a crude membrane fraction of embryonic chick neural retinas which had been iodinated in the absence of Ca^{2+} (a) and medium (b) conditioned for 12 h by such iodinated retinas. Gp130 and gp90 are indicated by arrowheads in panels a and b, respectively.

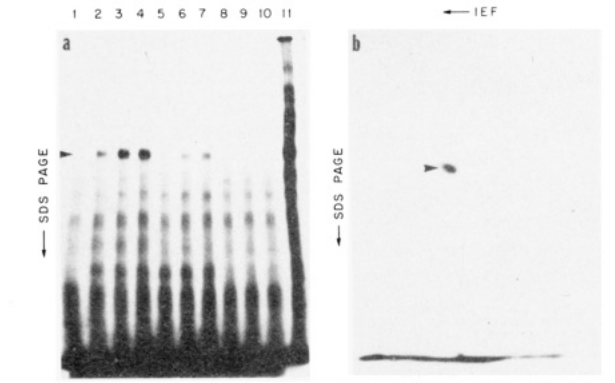

FIGURE 2: Tryptic digestion of medium conditioned for 8 h by embryonic chick neural retinas which were iodinated in the absence of Ca^{2+}. SDS–PAGE (a) of undigested medium (lane 11), medium digested in the absence of divalent cations (lane 1), and medium digested in the presence of 1, 5, and 10 mM Ca^{2+} (lanes 2–4), 1, 5, and 10 mM Mn^{2+} (lanes 5–7), and 1, 5, and 10 mM Mg^{2+} (lanes 8–10). Two-dimensional gel (b) of a duplicate of the sample shown in panel a, lane 4.

Ca^{2+} Protects gp90 from Tryptic Cleavage. Since gp130 is resistant to tryptic cleavage in the presence of Ca^{2+}, we asked if this were similarly true of gp90. As expected, tryptic digestion of medium conditioned by iodinated retinas eliminates many of the labeled proteins (Figure 2a, compare lanes 1 and 11). The inclusion of Ca^{2+} during trypsinization, however, partially protects labeled molecules of $M_r \sim 9 \times 10^4$ (Figure 2a, lanes 2–4) which are, by two-dimensional analysis, gp90 (Figure 2b). Mn^{2+} is also able to protect gp90 but is less effective even at higher concentrations (Figure 2a, lanes 5–7); Mg^{2+} is ineffective at the highest concentration tested (Figure 2a, lanes 8–10).

Tryptic Peptide Maps of Labeled gp130 and gp90 Derived from Iodinated Retinas Are Very Similar. The above data are consistent with the hypothesis that gp90 is a fragment of cell surface gp130 which is released into the medium during in vitro culture. In order to test this hypothesis more directly, we obtained labeled gp130 and gp90 from two-dimensional gels of, respectively, iodinated retinas (Figure 1a) and medium conditioned by them (Figure 1b) and subjected both to two-dimensional tryptic peptide mapping. The maps of gp130 (Figure 3a) and gp90 (Figure 3b) are very similar; indeed, they would be indistinguishable but for the absence of peptide A from the map of gp90 and the virtual absence of peptide B

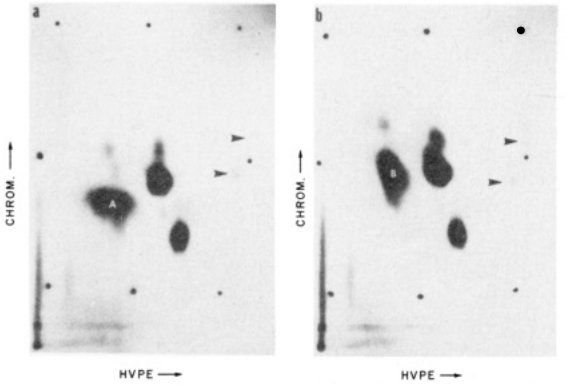

FIGURE 3: Two-dimensional tryptic peptide maps of gp130 (a) and gp90 (b) derived from, respectively, the gels shown in Figure 1a,b. The arrowheads indicate faint spots that may not be detectable in the figure. The spots labeled A and B are discussed in the text.

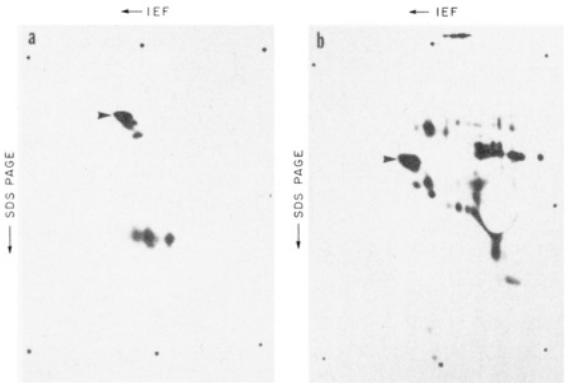

FIGURE 4: Two-dimensional gels of the NP-40 insoluble residue of iodinated embryonic retinas (a) and retina-conditioned medium enriched for gp90 by gel filtration and affinity chromatography on Con A–agarose (b). Gp130 and gp90 are indicated by arrowheads in panels a and b, respectively.

from the map of gp130. Given the similarity of the above peptide maps and the fact that gp90, like gp130, is protected from tryptic cleavage by Ca^{2+}, but not by Mg^{2+}, gp130 is the most likely precursor of gp90.

Accepting for the moment that gp90 is a fragment of gp130, there must be a labeled peptide in the gp130 digest which corresponds to peptide B in the gp90 map. That this is peptide A, rather than some other labeled peptide not recovered from the gp130 digest, is suggested by the fact that recovery of labeled peptides at each step in the procedure is very similar from both gp130 and gp90 (data not shown) and, furthermore, that peptides A and B each represent a similar percentage of the total label applied.

Tryptic Peptide Maps of gp130 and gp90 Iodinated after Reduction and Denaturation Are Also Similar. A number of minor peptides, in particular, the two marked with arrowheads in Figure 3, are common to the maps of gp130 and gp90 derived from iodinated retinas, suggesting preferential labeling of residues in the major peptides. So as to ensure the efficient labeling of all iodinatable residues in gp130 and gp90 prior to peptide mapping, the proteins were reiodinated after 2D gel electrophoresis (i.e., after reduction and denaturation).

While gp130 and gp90 are major labeled species in, respectively, iodinated retinas and their conditioned medium, neither contributes substantially to the total protein. Therefore, we partially purified both proteins to avoid depending entirely on the two-dimensional gel system for their isolation. Gp130 was obtained from the NP-40 insoluble residue of iodinated retinas (Figure 4a) while retina-conditioned medium was enriched for gp90 by gel filtration and affinity chromatography on Con A–agarose (Figure 4b).

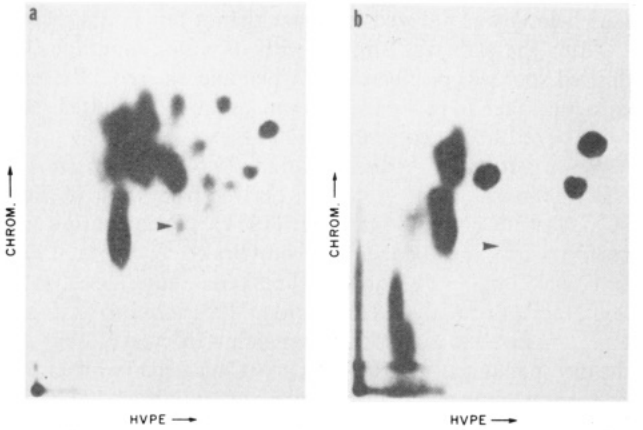

FIGURE 5: Two-dimensional tryptic peptide maps of gp130 (a) and gp90 (b) which were reiodinated after excision from respectively the gels shown in Figure 4a,b. The arrowheads indicate that spots that may not be visible in the figure.

Comparison of the two-dimensional tryptic peptide maps of reiodinated gp130 (Figure 5a) and gp90 (Figure 5b) reveals that they have in common all of the peptides common to the maps derived from iodinated retinas (Figure 3). However, the relative intensities of these common peptides depend on how the proteins are iodinated; two (three if one considers A and B homologous) peptides are labeled preferentially during the iodination of intact retinas while a second two (those marked with arrowheads in Figure 3) are labeled much more efficiently when the isolated proteins are reiodinated. Several peptides are unique to the map of reiodinated gp130 (Figure 5a) [i.e., absent from the map of reiodinated gp90 (Figure 5b) as well as the maps of gp130 and gp90 iodinated at the cell surface (compare Figure 5 with Figure 3)].

Thus, gp130 appears to contain two groups of iodinatable residues, those preferentially labeled at the cell surface and those labeled well only after denaturation.[1] HOI, the major iodinating species generated by Iodogen at low iodide concentration (J. C. Speck, Jr., personal communication), is a polar molecule, and its reactivity with iodinatable residues in cell surface gp130 may therefore be a measure of their exposure to solvent. Since many of the iodinatable residues of gp130 are labeled only after denaturation and are not present in gp90, they may be present in a cryptic portion of cell surface gp130. On the other hand, gp90 retains most, if not all, of the iodinatable residues of gp130 which are readily labeled during cell surface iodination and thus appears to be derived from a domain of gp130 which is exposed at the cell surface. Consistent with this hypothesis, the tryptic peptide maps of gp130 and gp90 derived from retinas cultured with [^{3}H]-glucosamine are virtually indistinguishable (data not shown).

Tryptic Peptide Map of gp90 Iodinated in Solution Is Very Similar to That of gp90 Derived from Iodinated Retinas. Since gp90 in solution is, like cell surface gp130, partially protected from tryptic cleavage by Ca^{2+}, it apparently shares certain conformational features with the portion of gp130 from which it is derived. The tryptic peptide map of gp90 iodinated in solution prior to denaturation (Figure 6) supports this hypothesis; this map is more similar to that of gp90 derived from

[1] Since reiodination of gp130 as described above routinely results in an increase in specific activity of ca. 10^2, label incorporated prior to reiodination has no significant effect on peptide intensities in the maps of reiodinated proteins.

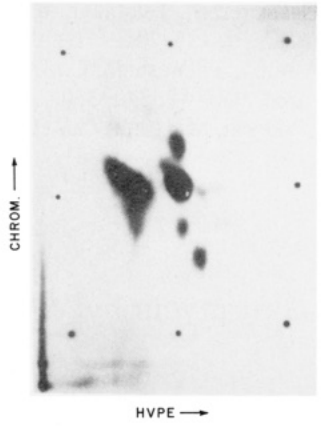

FIGURE 6: Two-dimensional tryptic peptide map of gp90 derived from a duplicate of the gel shown in Figure 4b and thus iodinated in solution prior to denaturation.

cultures of iodinated retinas (Figure 3b) than to the map of gp90 iodinated after denaturation (Figure 5b).

Discussion

The data presented here indicate that gp90 is a fragment of cell surface gp130 which is released into the medium during in vitro culture. Indeed, it would appear that gp90 comprises that portion of gp130 which is preferentially accessible to cell surface iodination and, further, that this portion of gp130 retains certain configurational features, as well as the ability to interact with Ca^{2+}, after its release from the cell surface as gp90. In addition, gp90 carries the major glucosamine-containing oligosaccharides of gp130. These characteristics would be expected if gp90 were the domain of gp130 exposed at the cell surface.

Other laboratories, working with different cell types, have identified cell surface proteins which bear striking similarities to gp130 and gp90. Takeichi (1977) first reported that the inclusion of Ca^{2+} during trypsinization of fibroblasts protects a cell surface protein of $M_r \sim 1.5 \times 10^5$. Recent evidence indicates that the M_r of this protein is closer to that of gp130 (M. Takeichi, personal communication).

The same approach was used to identify a cell surface component of $M_r \sim 1.24 \times 10^5$ on embryonal carcinoma cells, which is protected from tryptic digestion by Ca^{2+}. This molecule is recognized by rabbit antibodies whose Fab fragments inhibit Ca^{2+}-dependent intercellular adhesion (Yoshida & Takeichi, 1982). More recently, Fab fragments of a monoclonal antibody (ECCD-1) recognizing the same component along with a component of $M_r \sim 1.04 \times 10^5$ have also been shown to inhibit Ca^{2+}-dependent embryonal carcinoma cell adhesion (Ogou et al., 1983).

Embryonal carcinoma cells were also used by Hyafil et al. (1980) as the source of a Con A binding component of $M_r \sim 8.4 \times 10^4$ termed uvomorulin. Like gp90, uvomorulin is protected from tryptic cleavage by Ca^{2+} and, to a lesser extent, Mn^{2+}. Monoclonal antibodies to uvomorulin inhibit the Ca^{2+}-dependent compaction of early mouse embryos. This monoclonal antibody has recently been shown to recognize components with molecular weights identical with those recognized by ECCD-1 (M. Takeichi, personal communication) and, thus, uvomorulin may well be a fragment of the $M_r \sim 1.24 \times 10^5$ embryonal carcinoma molecule.

Gallin et al. (1983), working with embryonic chick liver, obtained a component of $M_r \sim 8.1 \times 10^4$ by a procedure very similar to that employed by Hyafil et al. in the preparation of uvomorulin (trypsinization of isolated membranes in the presence of Ca^{2+}). Fab fragments of polyclonal antibodies to this protein inhibit Ca^{2+}-dependent liver cell adhesion. These antibodies recognize components in liver cell membranes with molecular weights of ca. 1.24×10^5, 9.4×10^4, and 8.1×10^4. A mouse liver cell surface protein, also of $M_r \sim 1.24 \times 10^5$, has been shown to be stable to trypsinization in the presence of Ca^{2+} and implicated in Ca^{2+}-dependent adhesion (Ogou et al., 1983).

The relation of each of these cell surface proteins to Ca^{2+}-dependent adhesion has been based, at least in part, on immunological criteria; that is, Fab fragments of either monospecific (Hyafil et al., 1980; Gallin et al., 1983; Ogou et al., 1983) or polyspecific (Grunwald et al., 1982; Yoshida & Takeichi, 1982) antibodies inhibit adhesion. In each case where a polyspecific antiserum was used, a protein was partially purified which reversed the inhibition of adhesion as well as the immunoprecipitation of a cell surface protein of $M_r \sim 1.3 \times 10^5$. We have established in this report that gp90, the major immunoprecipitable component of the preparation which reverses the inhibition of adhesion and the immunoprecipitation of gp130, is indeed a fragment of gp130.

Previously published work from our group further strengthens the relationship of gp130 to Ca^{2+}-dependent adhesion among embryonic chick neural retina cells. In particular, gp130 is invariably present on cells capable of Ca^{2+}-dependent adhesion; this is true whether cells are prepared such that they have a functionally intact adhesive system (Cook & Lilien, 1982) or require a period of repair to recover the system (Geller & Lilien, 1983).

The striking similarities in the characteristics of the molecules identified in each of these systems suggest that they serve similar functions. It is thus tempting to speculate that they are members of a family of related cell surface proteins involved in Ca^{2+}-dependent intercellular adhesion. However, further work is needed to determine in more detail their similarities and differences.

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Registry No. Calcium, 7440-70-2; manganese, 7439-96-5; trypsin, 9002-07-7.

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Evidence for the Conversion of Adenosine to 2′-Deoxycoformycin by *Streptomyces antibioticus*†

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ABSTRACT: The incorporation and distribution of ^{14}C in 2′-deoxycoformycin, elaborated by *Streptomyces antibioticus*, were studied with [U-^{14}C]glycine, [U-^{14}C]adenosine and [U-^{14}C]adenine. Similar ratios of ^{14}C in the aglycon and carbohydrate portions of 2′-deoxycoformycin, ara-A, and adenosine isolated from the RNA indicated that [U-^{14}C]adenosine was incorporated into 2′-deoxycoformycin without cleavage of the N-glycosylic bond. Following the addition of [U-^{14}C]adenine, 98% of the ^{14}C isolated from [^{14}C]-2′-deoxycoformycin resided in the aglycon. 2′-Deoxycoformycin biosynthesis may not require the de novo purine biosynthetic pathway as evidenced by the failure to detect incorporation of [U-^{14}C]glycine into 2′-deoxycoformycin. These data suggest that the biosynthesis of 2′-deoxycoformycin involves the incorporation of the carbon–nitrogen skeleton of an intact purine nucleoside or nucleotide, thereby implying that a purine ring is opened enzymatically between C-6 and N-1 and a one-carbon unit is added to form the 1,3-diazepine ring of 2′-deoxycoformycin.

2′-Deoxycoformycin,[1] the potent, tight-binding inhibitor of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) having a $K_i = 2.5 \times 10^{-12}$ M (Agarwal et al., 1977), has been isolated from the culture filtrates of *Streptomyces antibioticus* NRRL 3238 (Woo et al., 1974; Ryder et al., 1975; Dion et al., 1977). This nucleoside and its D-*ribo* analogue, coformycin, represent the two most potent inhibitors known for adenosine deaminase (Suhadolnik, 1979). The extremely tight-binding inhibitory properties are attributed, in part, to the unique heterocyclic ring system that structurally is very similar to the sp^3 transition state (Wolfenden, 1972) believed to be involved in the deamination process (Figure 1). The structure of 2′-deoxycoformycin was unequivocally established via single-crystal X-ray analysis by Woo et al. (1974) as (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol. Baker and co-workers have developed synthetic routes for this nucleoside and its congeners (Chan et al., 1982; D. C. Baker et al., unpublished results), establishing that the 8*R* isomers are the tight-binding entities among possible diastereomers. 2′-Deoxycoformycin has attracted considerable interest, as the inhibition of adenosine deaminase is of importance in the areas of both cancer and virus chemotherapy and in immunosuppression (Agarwal, 1982). 2′-Deoxycoformycin, both alone and in combination with ara-A, is at present under phase I and II clinical trials against a number of neoplasms, with dramatic results being observed in some patients with refractory T-cell disease (Prentice et al., 1980; Hershfield et al., 1983).

The object of the study reported herein is to investigate the biosynthesis of 2′-deoxycoformycin, particularly as it pertains to the origin of the fused 5,7-membered heterocyclic aglycon which contains an additional methylene group located between the N-1 and C-6 in a typical purine skeleton (see Figure 1). While 1,4-diazepines such as anthramycin have been established as being derived from L-tyrosine and L-tryptophan by Hurley & Gairola (1979), there is no information about the biosynthetic origins of the 1,3-diazepines such as 2′-deoxycoformycin. An additional attractive feature to these studies is the fact that another important nucleoside antibiotic, 9-β-D-arabinofuranosyladenine (ara-A), is produced concomitantly with 2′-deoxycoformycin and is in fact the major product of the *S. antibioticus* fermentation. Earlier studies on the epimerization of the C-2′ hydroxyl group of adenosine suggest that adenosine is directly converted to ara-A (Farmer & Suhadolnik, 1972), and more recently R. J. Suhadolnik et al. (R. J. Suhadolnik, J. M. Wu, M. M. Anderson, D. von Minden, and J. A. McCloskey, unpublished results) have reported the isolation and partial purification of an adenosine 2′-epimerase that catalyzes a 10% in vitro conversion of adenosine to ara-A. Perhaps the biosynthesis of 2′-deoxycoformycin might be a process closely related to the conversion of adenosine to ara-A or conceivably a process that could involve a common biosynthetic intermediate for the biosynthesis of both ara-A and 2′-deoxycoformycin. This paper provides evidence that adenosine and a one-carbon unit are the direct precursors of 2′-deoxycoformycin.

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[1] Abbreviations: ara-A, 9-β-D-arabinofuranosyladenine; AMP, adenosine 5′-monophosphate. Other names for 2′-deoxycoformycin (dCF) include co-vidarabine (CoV) and pentostatin (USAN). The chemical name is (8*R*)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol.

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