Presence of Laminin B Chain–Like Protein in Bovine and Rat Adrenal Chromaffin Granules

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The presence of a glycoprotein laminin in bovine adrenal chromaffin granules was examined by SDS-PAGE followed by immunoblotting. The two chromaffin granule membrane fractions were obtained by linear sucrose gradient centrifugation followed by freezing and thawing and gel-filtration of the chromaffin granule-rich fraction, respectively. The purity of the granules in these fractions was examined by electron microscopy. These fractions contained laminin B chain–like immunoreactivity as a major immunoreactive component against anti-laminin. Laminin A chain–like immunoreactive protein was undetectable. The soluble fraction of the chromaffin granules contained no immunoreactive peptide. The presence of laminin-like immunoreactivity in the chromaffin granules was confirmed by immunocytochemical study. Laminin B chain–like immunoreactivity was also identified in the rat adrenal chromaffin granule fraction. Laminin A chain was hardly detected, as in the case of bovine adrenals. Structure of laminin in chromaffin granules in bovine and rat adrenals may be different from that of mouse Englebrethe-Holm-Swarm sarcoma laminin. The functional significance of laminin B chain–like protein in the granules is unknown at present.

Key words: adrenal medulla, bovine, chromaffin granule, laminin B chain, rat.

The glycoprotein laminin, a component of basement membranes in various tissues (1), has been found to contribute to cell adhesion (1, 2), cell migration (3, 4), tumor cell invasion through extracellular matrices (5), neurite outgrowth (6–8), and cell differentiation (9–11). Laminin is composed of one A chain (apparent molecular weight on SDS-PAGE of about 400 kDa), one B1 and one B2 chain (apparent molecular weight of about 215 and 205 kDa, respectively) (12), and is cruciform with three short arms and one long arm (13).

Laminin has been detected in the basement membranes in various tissues. However, recent immunocytochemical studies have revealed the presence of laminin in intracellular organelles such as lysosomes and rough endoplasmic reticulum (rER) in the central nervous system of mouse and rat (14). Laminin was also detected in rER and in the inner walls of Golgi apparatus of rat anterior pituitary (15, 16). Laminin in rER and Golgi apparatus is probably concerned with intracellular laminin production. Laminin may be degraded in lysosomes after phagocytosis. In addition, immunocytochemical studies have demonstrated the presence of laminin in the secretory granules of rat anterior pituitary (15–17) and in gonadotrophs in mouse pituitary (18). In these reports, the absence of laminin A chain in the granules was demonstrated by immunoblotting (17, 18). The role of laminin in these secretory granules is unknown at present.

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Abbreviations: EHS, Englebrethe-Holm-Swarm; EGTA, ethylene bis(oxyethylenenitriilo)tetraacetic acid, MES, 2-(N-morpholino)ethane sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; rER, rough endoplasmic reticulum.

Adrenal chromaffin granules, catecholamine- and neuropeptide-storing organelles, have been extensively studied as a model system for secretory granule exocytosis. If laminin is also present in the granules, this system could be a tool to study the role of laminin in the secretory granules. In the present study, laminin B chain–like immunoreactivity was identified in bovine and rat adrenal chromaffin granule fractions by immunoblotting, using the laminin-specific polyclonal antibody against mouse Englebrethe-Holm-Swarm sarcoma laminin (17). The presence of laminin-like protein in the granules was confirmed by immunocytochemical study.

MATERIALS AND METHODS

Isolation of the Bovine and Rat Adrenal Chromaffin Granule Fractions—Bovine adrenals were obtained from a local slaughterhouse. Eight-week-old Wistar-Imamichi male rats were used. Adrenal medulla was homogenized in the medium composed of 0.3 M sucrose, 20 mM 2-(N-morpholino)ethane sulfonic acid (MES)–KOH buffer (pH 5.9), 2 mM ethylene bis(oxyethylenenitriilo)tetraacetic acid (EGTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). For rats, whole adrenals were used in place of adrenal medulla. The homogenate was centrifuged at 1,500×g for 5 min and the supernatant was recentrifuged at 10,000×g for 20 min. The sediment was suspended in a small amount of the homogenizing medium, layered on a linear sucrose concentration gradient from 1.0 to 2.0 M for bovine and 0.8 to 1.6 M for rat, and centrifuged at 75,000×g for 1 h. In the case of bovine adrenals, the fraction containing chromaffin granules was lysed by freezing and

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thawing in a hypotonic medium consisted of 2 mM EGTA and 1 mM PMSF. This lysate was centrifuged at 108,000×g for 1 h. The resultant supernatant was dialyzed against distilled water overnight and lyophilized. The lyophilized sample and the precipitate were suspended in a small amount of distilled water and used as the soluble fraction of the chromaffin granules and the chromaffin granule membrane fraction, respectively. A bovine chromaffin granule membrane fraction that was hardly contaminated by rER-like debris was also prepared by the method of Phillips (19). All procedures described above were carried out at 4°C.

**SDS-PAGE**—SDS-PAGE was performed by the method of Laemmli (20) using a 5 to 15% linear concentration gradient polyacrylamide gel with a 4% stacking gel. In some experiments, SDS-PAGE was carried out using Phast System (Pharmacia LKB Biotechnology, Uppsala, Sweden) with a 4 to 15% linear concentration gradient polyacrylamide gel. After electrophoresis, proteins in the gel were electrophoretically blotted to a polyvinylidene difluoride membrane by the method of Burnett (21). Immunostaining was carried out using a commercially available kit (Protoblot, Promega, Wisconsin, U.S.A.) with rabbit anti-EHS laminin serum.

**Electron Microscopy and Immunoelectron Microscopy**—The bovine chromaffin granule fraction and the chromaffin granule membrane fraction were suspended in a small amount of the homogenizing medium and pre-fixed in 5% glutaraldehyde containing 0.1 M sodium cacodylate buffer, pH 7.4, and 2 mM CaCl₂ at 4°C for 60 min, then washed twice with the cacodylate buffer for 10 min. The samples were post-fixed with 1% OsO₄ in the cacodylate buffer for 60 min and washed twice with the buffer for 10 min. Ultrathin sections (100 nm thick) were stained with uranyl acetate and lead citrate.

For immunocytochemistry, small segments of fresh bovine adrenal medulla were fixed by immersion in 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C for 30 min. After fixation, tissues were incubated in 0.05 M ammonium chloride in phosphate-buffered saline (PBS), pH 7.4, for 2 h at room temperature to block free aldehyde groups. The tissues were then rinsed in the cacodylate buffer for 1 h. The resultant supernatant was dialyzed against mouse EHS sarcoma laminin was from L.S.L., Tokyo. Colloidal gold-labeled secondary goat antibody was from Amersham Int. plc, Amersham, U.K.

**RESULTS**

**Fractionation of Chromaffin Granules from Bovine Adrenal Medulla**—The bovine adrenal chromaffin granule fraction was obtained by sucrose gradient centrifugation. As shown in Fig. 1, the concentrations of adrenaline, noradrenaline, ATP, and ATP, the major chromaffin granule soluble components, were high in the fraction obtained at sucrose concentration of about 1.9 M. Figure 2a shows an electron micrograph of this fraction (fraction No. 5). Although the major component of the fraction was the chromaffin granules, an appreciable quantity of debris was present that was probably derived from rER. Golgi apparatus, lysosomes, mitochondria, and nucleus were scarcely found. This fraction contained two types of chromaffin granules: a smaller number of electron dense granules

Determination of Catecholamines, ATP, and Protein—Catecholamines were analyzed by HPLC using a reverse phase column (LiChrospher 100, 4×240 mm, E. Merck, Darmstadt, Germany) with a fluorescent spectrophotometer equipped with a flow cell (RF-5000, Shimadzu, Kyoto). ATP was also analyzed by HPLC using a strong cation exchange column (Radial-Pak SAX, 8×100 mm, Whatman, Maidstone, England, packed by Waters Associates, Massachusetts, U.S.A.) and detected by absorbance at 260 nm. Protein concentration was measured by the method of Bradford (22) with bovine gamma globulin as a standard.

**Chemicals**—EGTA, MES, mouse EHS laminin, and PMSF were purchased from Sigma Chem., Missouri, U.S.A. Acrylamide, molecular weight markers for SDS-PAGE and the dye reagent for protein determination were from Bio-Rad, California, U.S.A. Rabbit antiserum directed against mouse EHS sarcoma laminin was from L.S.L., Tokyo. Colloidal gold-labeled secondary goat antibody was from Amersham Int. plc, Amersham, U.K.
Fig. 2. Electron micrographs of the bovine adrenal chromaffin granule fraction and the mitochondria-rich fraction. a: Fraction No. 5 indicated in Fig. 1. Arrows, electron-dense granules; arrowheads, moderately electron-dense granules b: Fraction No. 20 indicated in Fig. 1. Bars 0.5 μm ×34,000.

TABLE I. Catecholamine concentration in fractions obtained from the homogenate of bovine adrenal medulla. Values are the mean of two separate experiments.

<table>
<thead>
<tr>
<th>Source</th>
<th>Catecholamine concentration (nmol/mg protein)</th>
<th>Adrenaline</th>
<th>Noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td></td>
<td>108</td>
<td>59</td>
</tr>
<tr>
<td>1,500 x g Supernatant</td>
<td></td>
<td>107</td>
<td>59</td>
</tr>
<tr>
<td>10,000 x g Precipitate</td>
<td></td>
<td>203</td>
<td>101</td>
</tr>
<tr>
<td>Chromaffin granule fraction</td>
<td></td>
<td>542</td>
<td>271</td>
</tr>
<tr>
<td>Chromaffin granule lysate</td>
<td></td>
<td>462</td>
<td>235</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td></td>
<td>59</td>
<td>37</td>
</tr>
<tr>
<td>Membrane fraction</td>
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(arrows) and a larger number of less electron dense ones (arrowheads).

Fraction No. 5 was lysed by freezing and thawing in a hypotonic medium, and the soluble and membrane fractions of the granules were obtained by centrifugation. As indicated in Table I, the concentration of adrenaline and noradrenaline in the soluble fraction was about 4 times higher than that of the homogenate. Other amines such as dopamine were hardly detected by HPLC in the all fractions shown in Table I. The concentration ratio of adrenaline to noradrenaline in the soluble fraction was almost similar to that in the homogenate. The concentration ratio of catecholamines (adrenaline plus noradrenaline) to ATP in the soluble fraction was about 4:1, which is the established concentration ratio of the bovine adrenal chromaffin granules.

As shown in Fig. 2b, fraction No. 20, obtained at about 1.45 M sucrose, contained mitochondria as a major component. In this fraction a small number of electron-dense granules and lysosomes were present. As indicated in Fig. 1, this fraction was poor in catecholamines and ATP.

A chromaffin granule membrane fraction was also obtained by a different method (19) involving gel-filtration of the chromaffin granule-rich fraction in hypotonic medium. As indicated in Fig. 3, this fraction was hardly contaminated by rER-like debris and organelles other than chromaffin granules.

**Immunoblotting Using Antiserum against Mouse EHS Sarcoma Laminin**—The homogenate of adrenal medulla and the membrane and soluble fractions derived from the
fraction No. 5 were applied to SDS-PAGE followed by immunoblotting using an antiserum directed against mouse EHS sarcoma laminin in a 1:8,000 dilution ratio. As shown in Fig. 4, lane 2, mouse EHS laminin B1 and B2 chains co-migrated, resulting in a broad band. In the present SDS-PAGE system, B1 and B2 chains could not be separated from each other. Laminin immunoreactive protein bands were detected in the homogenate of adrenal medulla (Fig. 4, lane 1). The major immunoreactive component migrated to the position corresponding to EHS laminin B chain. Laminin A chain–like immunoreactive band was not detectably stained. Many immunoreactive protein bands that exhibited smaller apparent molecular weights than laminin B chain were also present. In the chromaffin granule membrane fraction, laminin B chain–like protein was present as a major immunoreactive component (Fig. 4, lane 3). Antiserum was used at a 1:8,000 dilution ratio.
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Fig. 5. Immunoelectron micrographs of bovine adrenal chromaflin cells in which sites of laminin immunoreactivity are visualized with a secondary antibody coupled to 10 nm colloidal gold particles. a: Anti-EHS laminin was used at a dilution ratio ×1,000 as a primary antibody. Arrows, gold particles distributed on the edge of the chromaflin granules; arrowheads, gold particles distributed on the rER-like structure. b: Control serum was used. Bars 0.5 μm. ×46,000.

Immunocytochemical Study on Chromaflin Granules—Figure 5a represents an immunoelectron micrograph, prepared with an antiserum against EHS laminin, of the bovine adrenal chromaflin cells. Laminin immunoreactivity was detected over the chromaflin granules. In some cases, colloidal gold particles were distributed at the edge of the granules (arrows in Fig. 5a). Although rER could not be visualized clearly because of the fixation for immunocytochemistry, several gold particles were observed on the rER-like structure (arrowheads in Fig. 5a). When non-immunized serum was used in place of the antiserum at the same dilution ratio, gold particles were undetectable over the field of the electron micrograph (Fig. 5b).

Laminin B Chain-Like Protein in the Rat Chromaflin Granule Fraction—As shown in Fig. 6, the rat adrenal chromaflin granule fraction was obtained at about 1.35 M sucrose concentration by linear sucrose gradient centrifugation (fraction No. 6). This concentration was significantly lower than that in the bovine chromaflin granule fraction.

As shown in Fig. 7, lane 1, laminin subunit-like immunoreactive bands were present in the homogenate of rat adrenals. The major immunoreactive component was laminin B chain-like protein. Two immunoreactive bands, moving significantly slower and slightly faster than A chain, were also present as minor bands. Their apparent molecular weights were about 540 and 370 kDa, respectively. Laminin A chain-like protein band was undetectable in the homogenate. Although laminin B chain-like immunoreactivity was present in the chromaflin granule fraction as a major immunoreactive component (Fig. 7, lane 2), laminin A chain-like immunoreactive protein band was hardly detectable in this fraction. When control serum was used at the same dilution ratio in the antiserum (1:1,600), no band was developed in these samples.
Fig. 6. Separation of the rat adrenal chromaffin granule fraction by linear sucrose gradient centrifugation. The 10,000×g precipitate fraction, prepared from 10 mg of protein of the adrenal homogenate, was centrifuged on the linear concentration gradient of sucrose from 0.8 to 1.6 M and fractions of 0.25 ml were taken.

**Fig. 7.** Presence of laminin B chain-like protein in the rat adrenal chromaffin granule fraction. SDS-PAGE was carried out using the Phast System. Anti-EHS laminin serum was used at a 1:1,600 dilution ratio. Lane 1, 2 μg of protein of the adrenal homogenate; and lane 2, 0.78 μg of protein of the chromaffin granule fraction. A, laminin A chain; B, laminin B chain.

**DISCUSSION**

In the present study, laminin B chain-like immunoreactivity was detected in the bovine adrenal chromaffin granule membrane fraction and in the rat chromaffin granule fraction as a major immunoreactive component against antibody directed to mouse EHS sarcoma laminin. The presence of laminin in the bovine chromaffin granules was confirmed by immunocytochemical study. In these fractions, laminin A chain-like immunoreactivity was not detected. The bovine and rat adrenal homogenates also lacked laminin A chain-like immunoreactive protein. The major component of the immunoreactive proteins in the homogenates was laminin B chain-like protein. Non-stoichiometric expression of laminin subunits has been reported in mouse embryos (23, 24) and in rat liver epithelial cells (25). Laminin in these tissues as well as the adrenal medulla and the chromaffin granules of bovine and rat origin may not have the cruciform structure.

The bovine chromaffin granule fraction prepared by sucrose gradient centrifugation was contaminated by an appreciable amount of rER-like debris. The chromaffin granule membrane fraction was prepared from this fraction by freezing and thawing followed by centrifugation and is, therefore, assumed to contain a significant amount of rER-like component. In the immunocytochemical study, several gold particles were distributed on the rER-like structure. The possibility that laminin B chain-like immunoreactive protein is present in rER as well as in the chromaffin granules cannot be excluded by immunoblotting and immunocytochemistry. Immunocytochemical study has indicated the presence of laminin in rER in the central nervous system and anterior pituitary of rat (14–16).

Immunohistochemical study has revealed that chromaffin cell clusters in calf adrenal medulla are surrounded by laminin immunoreactive extracellular matrices (26). In the present study the content of B chain-like protein in the chromaffin granule membrane fractions seemed low in the immunoblotting, when compared to that in the homogenate. This is probably due to the presence of extracellular matrices in the homogenate.

Electron microscopic observation has shown noradrenaline granules to be stained more densely by uranyl acetate and lead citrate than adrenaline granules (27). In the present study, the bovine chromaffin granule fraction contained a smaller number of electron-dense granules and a larger number of less electron-dense ones. Furthermore, the concentration ratio of adrenaline to noradrenaline in the soluble fraction derived from the bovine chromaffin granule fraction was similar to that in the homogenate. The chromaffin granule membrane fraction used in the immunoblotting probably contained both types of the granule membrane. In the immunocytochemical study, adrenaline cells could not be distinguished from noradrenaline cells owing to weak staining by uranyl acetate and lead citrate caused by the fixation for immunocytochemistry. Therefore, it is not clear at present whether laminin B chain-like protein is distributed in both types of granules.

In PC12 cells, derived from rat pheochromocytoma cells, laminin is secreted as a soluble protein through a pathway...
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distinct from that of catecholamine output (28). In the present study laminin B chain–like protein was identified in the membrane-bound form. Therefore, this protein may not be concerned with the transport of laminin to the extracellular matrices. In cultured calf adrenal chromaffin cells, laminin added to the medium has been observed to increase both levels and activity of tyrosine hydroxylase (26), one of the catecholamine synthesizing enzymes. Laminin B chain–like protein found in the chromaffin granule membrane may regulate tyrosine hydroxylase activity after exposure to the cell surface as a result of exocytosis. On the other hand, the presence of a cytoplasmic receptor of elastin/laminin has been proposed (29). Laminin B chain–like protein in the chromaffin granule membrane may interact with such a receptor in the cytoplasm. Although the role of laminin B chain–like protein in the chromaffin granule membrane is unknown at present, this protein may be involved in the function of the chromaffin granules.

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