are very different from the data collected using electron microscopy of negatively stained gap junctions (5). In Fig. 2, the x-ray intensities are plotted along with the approximate electron microscope measurements. The x-ray data have substantially higher frequency detail along lattice lines than the electron microscope data because the principal contrast seen in the electron microscope is the 30-Å thick layer of stain in the extracellular gap, whereas the principal contrast for x-rays is the 150-Å thick protein unit. In Fig. 3, a single section of a three-dimensional electron density map of a gap junction specimen is presented. This section is perpendicular to the membrane plane and cuts through the centers of two connexons. The cytoplasm is at the top and the center of the gap is at the bottom. The channel through the center of the connexon can be seen extending most of the way from the center of the gap to the cytoplasmic surface. The channel may not extend entirely through the junction to the cytoplasm. Analysis of diffraction patterns from isolated gap junctions in 50% sucrose shows that the sucrose fills the extracellular gap but fails to enter the channel. It is possible that the channel is closed at both cytoplasmic surfaces, excluding sucrose. This suggests that the isolated junctions are in a high resistance state.

Received for publication 18 May 1981.

REFERENCES

1. Caspar, D. L. D., D. A. Goodenough, L. Makowski, and W. C. Phillips. 1977. Gap junction structures. I. Correlated electron microscopy and x-ray diffraction. *J. Cell Biol.* 74:605.
2. Fallon, R. F., and D. A. Goodenough. 1981. Bulk isolation of mouse hepatocyte gap junctions. *J. Cell Biol.* In press.
3. Makowski, L., D. L. D. Caspar, W. C. Phillips, and D. A. Goodenough. 1977. Gap junction structures. II. Analysis of the x-ray diffraction data. *J. Cell Biol.* 74:629.
4. Makowski, L. 1978. Processing of x-ray diffraction data from partially oriented specimens. *J. Appl. Cryst.* 11:273.
5. Unwin, P. N. T., and G. Zampighi. 1980. Structure of the junctions between communicating cells. *Nature (Lond.).* 283:545.

ON THE STRUCTURAL ROLE OF THE ε-(γ-GLUTAMYL)LYSINE CROSS-LINK IN THE CELL MEMBRANE

R. B. HAUGLAND, T-I. LIN, R. M. DOWBEN, AND P. J. BIRCKBICHLER
Department of Pathology, Baylor University Medical Center, Dallas, Texas 75246 and Biomedical Division, S. Roberts Nobel Foundation, Ardmore, Oklahoma 73401 U.S.A.

We were first to show (1) that membrane proteins obtained from tissue-cultured cells contain significant amounts of isopeptide bonds between the ε-amino groups of lysine and the γ-carboxyl groups of glutamate. These bonds are formed as cross-links between membrane proteins by a Ca^{2+}-activated transglutaminase similar to blood clotting factor XIII present in plasma. Rigid structures such as wool and hair are quite rich in Glu-Lys isopeptide bonds (2). Nonproliferating cell membranes contain more isopeptide bonds than those of actively dividing cells, suggesting that one function of Glu-Lys is to stabilize the cell membrane and contribute to the termination of cell division (3).

Staining nonproliferating fibroblasts with transglutaminase antibodies (4) showed that transglutaminase is a membrane-associated enzyme. Thus, it is possible that Glu-Lys cross-links are involved in maintaining the "architecture" of the cell membrane in relation to cell shape and function.

MATERIALS AND METHODS

To investigate these possibilities, we grew L cells (NTC 929) or maintained surviving cultures of mouse ascites cells (Lettre) in the presence of one of two alternative substrates for the transglutaminase that prevent Glu-Lys cross-linking, cystamine or dansylcadaverine. Experiments were also performed in the presence of the Ca^{2+} ionophore A_{23187} which enhances cross-linking by increasing the concentration of Ca^{2+} intracellularly.

The structure of the cell membrane was studied four with fluorescent probes: 12-(9-anthroyloxy)stearic acid (12-AS); 2-(9-anthroyloxy)stearic acid (2-AS); 1,6-diphenyl-1,3,5-hexatriene (DPH); and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH). Fluorescence polarization and lifetime measurements were used to compare quantitatively the localized environment of the cell membrane under various conditions (5). Cell membrane fragments were prepared by nitrogen cavitation (6). Na^+,K^+-activated ATPase activity was determined in the presence and absence of 0.1 mM ouabain by a NADH oxidation-linked system (7). Glu-Lys was measured as described by Birckbichler et al. (3). Cholesterol was assayed using a kit supplied by Abbott Diagnostics (S. Pasadena, CA). Fluorescence polarization was measured with a photon counting system, and fluorescence lifetimes by a nanosecond pulse fluorimeter using single photon counting, both instruments constructed in our laboratory. The probes were used at a molar ratio of ~1:200 total lipid.

RESULTS

We observed a time-related decrease in the Glu-Lys content of membranes of cells grown in the presence of 0.1 mM dansylcadaverine or 1.0 mM cystamine (Fig. 1). A slight increase of Glu-Lys was found in cells treated with 0.5×10^{-6} M A_{23187}, a smaller increase than other investigators found in red blood cells (5), but we could not use

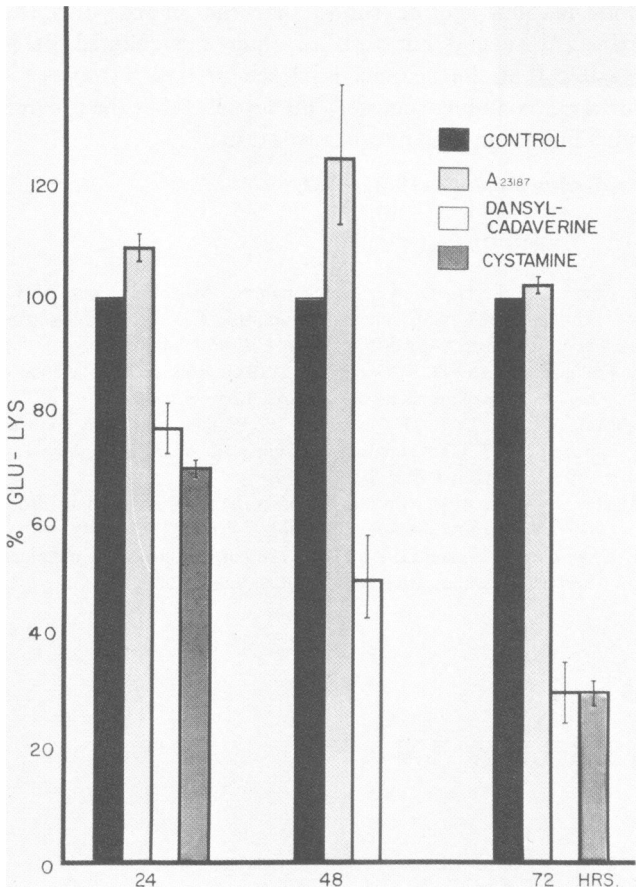

FIGURE 1 Glu-Lys content of L cell membranes (picomole/micromole) under the experimental conditions described in the text expressed as a percentage of control values.

such high concentrations of A_{23187} in our experiments because of its toxicity for our tissue-cultured cells. The decrease in isopeptide bonds in the membranes of dansylcadaverine or cystamine-treated L cells or ascites cells correlated with as much as a 50% decrease in NA^+, K^+-ativated ATPase activity and a 20% decrease in membrane cholesterol.

Fluorescence polarization (Table I) and fluorescence lifetimes (Table II) for the fluidity-sensitive probes 2-AS, 12-AS, DPH, and TMA-DPH showed a decrease in *P* values and a corresponding increase in lifetimes in membranes from cells grown with alternative transglutaminase substrates. These results correlate with the decreased amounts of Glu-Lys. The changes in fluidity take place at various depths in the lipid bilayer, because the changes for 2-AS and 12-AS were similar, although their chromophore is attached to different locations on the fatty acid hydrocarbon chain (5). A small increase in membrane fluidity was also found in the ionophore-treated group, possibly owing to the fact that this compound, like other membrane-active agents, may directly perturb the lipid components. The change in fluidity estimated from the use of DPH was similar to those obtained using 2-AS and 12-AS, but the polarization of TMA-DPH did not change under conditions that altered the amount of isopeptide bonds. The positive charge of TMA-DPH may anchor this new probe and considerably restrain its freedom of movement in the membrane.

TABLE I
FLUORESCENCE POLARIZATION (21°) IN L CELL MEMBRANES*

Probe	2-AS	12-AS	DPH	TMA-DPH
Group		*P*		
Control	0.193 ± 0.004	0.167 ± 0.003	0.330 ± 0.003	0.377 ± 0.007
A_{23187}	0.191 ± 0.004	0.161 ± 0.003	0.321 ± 0.001	0.367 ± 0.008
Cystamine	0.182 ± 0.002	0.149 ± 0.004	0.312 ± 0.004	0.356 ± 0.005
Dansyl-cadaverine	0.181 ± 0.003	0.157 ± 0.004	0.315 ± 0.002	0.369 ± 0.005

*The cells were grown for 72 h in the presence of (Ca^{2+}) transglutaminase alternative substrates or Ca^{2+} ionophore A_{23187}.

TABLE II
FLUORESCENCE LIFETIME (21°) OF 12-AS AND DPH*

Probe	12 AS		DPH	
Group	L cell membranes		Ascites Membranes	
	τ	Ramp	τ	Ramp
Control	2.32 ± 0.34	0.068	10.48 ± 0.01	0.84
	12.95 ± 0.14	0.612	3.16 ± 0.15	0.16
	7.90 ± 0.32	0.330		
A_{23187}	2.24 ± 0.01	0.068	10.54 ± 0.02	0.83
	13.24 ± 0.02	0.699	3.60 ± 0.15	0.17
	7.95 ± 0.15	0.233		
Cystamine	2.64 ± 0.42	0.091	10.64 ± 0.03	0.82
	13.85 ± 0.06	0.658	3.64 ± 0.14	0.18
	8.52 ± 0.03	0.251		
Dansyl-Cadaverine	1.90 ± 0.15	0.077	10.96 ± 0.05	0.78
	13.31 ± 0.03	0.682	4.74 ± 0.44	0.22
	7.76 ± 0.12	0.241		

*L cells were grown for 72 h and ascites cells were maintained for 24 h in the presence of transglutaminase alternative substrates or A_{23187}.

Because the fluorescent probes we used are regarded to be sensitive to changes in fluidity of the lipid portions of the bilayer, we studied the cholesterol content of the membranes and differences in cholesterol content that correlated with the fluorescence changes. Analysis of the fatty acid composition of membrane phospholipids is in progress.

CONCLUSION

Cells grown in the presence of 1.0 mM cystamine or 0.1 mM dansylcadaverine have fewer Glu-Lys cross-links in the membrane proteins, lower cholesterol content of the membranes, and increased membrane fluidity, as evaluated by the use of fluorescent probes.

Supported in part by contract F33615-80-C-0602 from the School of Aerospace Medicine, and grant PCM78-09313 from the National Science Foundation.

Received for publication 29 April 1981.

REFERENCES

1. Birckbichler, P. J., R. M. Dowben, S. Metacic, and A. G. Loewy. 1973. Isopeptide bonds in membrane proteins from eukaryotic cells. *Biochim. Biophys. Acta.* 291:149–155.
2. Metacic, S., and A. G. Loewy. 1979. Presence of the ε-(γ-glutamyl)lysine crosslink in cellular proteins. *Biochim. Biophys. Acta.* 576:263–268.
3. Birckbichler, P. J., H. A. Carter, J. R. Orr, E. Conway, and M. K. Patterson. 1978. ε-(γ-glutamyl)lysine isopeptide bonds in normal and virus transformed human fibroblasts. *Biochem. Biophys. Res. Commun.* 84:232–237.
4. Birckbichler, P. J., and M. K. Patterson. 1980. Transglutaminase and ε-(γ-glutamyl)lysine isopeptide bonds in eukaryotic cells. *In* Tumor Cell Surfaces and Malignancy. R. D. Hynes and C. F. Fox, editors. Alan R. Liss, Inc., New York. 845–855.
5. Thulborn, K. R., and W. H. Sawyer. 1978. Properties and location of a set of fluorescent probes sensitive to the fluidity gradient of the lipid bilayer. *Biochim. Biophys. Acta.* 551:125–140.
6. Schmidt-Ullrich, R., D. H. F. Wallach, and F. D. G. David. 1976. Membranes of normal lymphocytes and lymphoid cells neoplastically transformed by simian virus 40. I. High yield purification of plasma membrane fragments. *J. Natl. Cancer Inst.* 76:1107–1116.
7. Barnett, R. E. 1970. Effects of monovalent cations on the ouabain inhibition of the sodium and potassium ion activated adenosine triphosphatase. *Biochemistry.* 9:4644–4648.

CHARACTERIZATION OF THE AMPHIPATHIC STRUCTURE OF γ-GLUTAMYLTRANSPEPTIDASE F13

THOMAS FRIELLE AND NORMAN P. CURTHOYS

Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 U.S.A.

Rat renal γ-glutamyltranspeptidase (γGT) is an intrinsic membrane glycoprotein which is composed of two nonidentical subunits. The hydrophobic domain responsible for its membrane interaction is associated with the large subunit. Amphipathic (TγGT) and hydrophilic (PγGT) forms of γGT were purified from brush border membranes following Triton X-100 solubilization and papain proteolysis, respectively (1). Amino acid analysis of the separated TγGT and PγGT subunits revealed no significant differences in the composition of the small subunits. However, the large subunit of the TγGT contained an additional 150 residues, 56% of which were hydrophobic. Only the TγGT form can be incorporated into unilamellar phosphatidylcholine (PC) vesicles (2). Reconstitution provided a convenient means to protect the hydrophobic domain from proteolytic degradation and to facilitate its isolation.

RESULTS AND DISCUSSION

Fully active, ^{3}H-labeled TγGT was prepared by reductive methylation (3) and incorporated into [^{14}C]PC vesicles. Reconstitution was evident from the coelution of [^{3}H]TγGT and the [^{14}C]PC vesicles observed during chromatography on Sepharose 4B (Fig. 1). The TγGT-vesicle association is unaffected by treatment with TPCK-trypsin, *Staphylococcus aureus* V8 protease, or thermolysin. In contrast, incubation with papain causes the release of 85–95% of the γGT activity. During Sepharose 4B chromatography, the solubilized activity elutes with an elution volume characteristic of PγGT (Fig. 1). The fractions containing the [^{14}C]PC vesicles retain 30–40% of the ^{3}H-labeled protein but only 5–15% of the γGT activity. ^{3}H-labeled material also elutes in the retention volume (V_R) of the column. When [^{3}H]PγGT and [^{14}C]PC vesicles are preincubated with papain and then chromatographed, no ^{3}H label elutes with the vesicles or in the V_R.

The subsequent fractionation of the vesicle-associated material was monitored by sodium salicylate fluorography of samples subjected to electrophoresis on a 20% polyacrylamide slab gel. As shown in Fig. 2, reductive methylation incorporates ^{3}H label into both subunits of TγGT (lane 1). The vesicle-associated protein obtained following papain treatment of reconstituted [^{3}H]TγGT is a mixture of residual [^{3}H]TγGT and a newly generated ^{3}H-labeled peptide which migrates slightly slower than the dye front (lane 2). The residual [^{3}H]TγGT and the ^{3}H-labeled peptide were resolved by chromatography on Sephadex LH-60 in organic solvents. The residual amphipathic [^{3}H]TγGT is not retained by the resin (lane 3), whereas the hydrophobic ^{3}H-labeled peptide is partially adsorbed and is recovered with a greater elution volume (lane 4). Fluorescamine and ^{3}H radioactivity profiles of the LH-60 column fractions were coincident, indicating the absence of any unlabeled peptides.

The ^{3}H-labeled material isolated from the V_R fractions of the Sepharose 4B column was further resolved by Sephadex G-25 chromatography in 8 M acetic acid. This fraction contains at least three fluorescamine positive peptides, two of which were ^{3}H-labeled. The fractionated peptides ranged between 400 and 1,200 in mol wt. Dansyl-Cl analysis failed to detect the presence of free amino acids. Each of the peptides is apparently a unique