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ROBERT J. ALPERN

STEVEN C. HEBERT

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Contributors

Numbers in parentheses indicate the page number(s) on which the contribution begins.

MAURO ABBATE, MD (2563) *Mario Negri Institute for Pharmacological Research, Bergamo, Italy*

DALE R. ABRAHAMSON, PhD (691), *Professor and Chair, Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas, USA*

MARCIN ADAMCZAK, MD (2537), *Department of Nephrology, Endocrinology, and Metabolic Diseases, Silesian Medical University, Katowice, Poland*

HORACIO J. ADROGUÉ, MD (1721), *Professor of Medicine, Baylor College of Medicine, Chief, Renal Section, The Methodist Hospital, Houston, Texas, USA*

SETH L. ALPER, MD, PhD (1499), *Professor of Medicine, Harvard Medical School, Molecular and Vascular Medicine Unit and Renal Division, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA*

ROBERT J. ALPERN, MD (1005, 1539, 1645, 1667), *Dean and Ensign Professor of Medicine, Yale University School of Medicine, New Haven, Connecticut, USA*

THOMAS E. ANDREOLI, MD (849), *Distinguished Professor, Department of Internal Medicine, Department of Physiology and Biophysics, University of Arkansas College of Medicine, Little Rock, Arkansas, USA*

ANITA C. APERIA, MD, PhD (443), *Professor of Pediatrics Karolinska Institutet, Department of Woman and Child Health, Astrid Lindgren Children's Hospital, Stockholm, Sweden*

MATTHEW A. BAILEY (425), *Center for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom*

DANIEL BATLLE, MD, FACP (2113), *Earle, del Greco, and Levin Professor of Nephrology/Hypertension Professor of Medicine Chief, Division of Nephrology/Hypertension, Northwestern University, Feinberg School of Medicine, Chicago, Illinois, USA*

MICHEL BAUM, MD (707), *Professor of Pediatrics and Medicine, University of Texas Southwestern Medical Center, Dallas, Texas, USA*

THERESA J. BERNDT, PhD (1989), *Departments of Internal Medicine, Physiology, and Bioengineering, Division of Nephrology and Hypertension, Mayo Clinic, Rochester, Minnesota, USA*

MARK O. BEVENSEE (1429), *University of Alabama at Birmingham, Birmingham, Alabama, USA*

JÜRG BIBER, PhD (1979), *Institute of Physiology, Centre for Integrative Human Physiology, University Zürich-Irchel, Zürich, Switzerland*

DANIEL G. BICHET, MD, MSc (1225), *Professor of Medicine and Physiology, Hôpital du Sacré-Coeur de Montréal, Université de Montréal, Montréal, Québec, Canada*

RENÉ J.M. BINDELS (1769), *Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*

ROLAND C. BLANTZ, MD (565), *Professor and Head; Division of Nephrology-Hypertension, University of California San Diego and VA San Diego Healthcare System, La Jolla, California, USA*

WALTER F. BORON, MD, PhD (1429, 1481), *Department of Cell and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

D. CRAIG BRATER, MD (2763), *Dean and Walter J. Daly Professor, Indiana University School of Medicine, Indianapolis, Indiana, USA*

JOSEPHINE P. BRIGGS, MD (589), *Senior Scientific Officer, Howard Hughes Medical Institute, Chevy Chase, Maryland, USA*

ALEX BROWN (1803), *Renal Division, Washington University School of Medicine, St. Louis, Missouri, USA*

NIGEL J. BRUNSKILL (979), *Professor of Renal Medicine, Department of Nephrology and Cell Physiology, Leicester General Hospital, University of Leicester, United Kingdom*

GERHARD BURCKHARDT (2045), *Abteilung Vegetative Physiologie und Pathophysiologie, Zentrum Physiologie und Pathophysiologie, Georg-August-Univesilat Göttingen, Göttingen, Germany*

GEOFFREY BURNSTOCK (425), *Department of Anatomy & Developmental Biology, University College London, London, United Kingdom*

LLOYD CANTLEY, MD (297), *Professor of Medicine and Professor of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

CHUNHUA CAO, MD, PhD (627), *Department of Medicine, University of Maryland at Baltimore, Baltimore, Maryland, USA*

GIOVAMBATTISTA CAPASSO, MD (979) *Chair of Nephrology, School of Medicine, Second University of Naples, Naples, Italy*

MICHAEL J. CAPLAN, MD, PhD (1, 2283), *Professor, Department of Cell and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

HUGH J. CARROLL, MD (275), *Professor of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York, USA*

LAURENCE CHAN, MD, DPhil(Oxon), FRCP (1203), *Professor of Medicine, Director, Transplant Nephrology, Division of Renal Diseases and Hypertension, University of Colorado Health Sciences Center, Denver, Colorado, USA*

MOONJA CHUNG-PARK, MD (2399), *Case Western Reserve University, Cleveland, Ohio*

FREDRIC L. COE, MD (1945), *Professor of Medicine and Physiology, Department of Medicine and Physiology, University of Chicago School of Medicine, Chicago, Illinois, USA*

THOMAS M. COFFMAN, MD (343), *James R. Clapp Professor of Medicine, Professor of Cell Biology and Immunology, Chief, Division of Nephrology Duke University and Durham VA Medical Centers, Durham, North Carolina, USA*

WAYNE D. COMPER, PhD, DSc (2081), *Professor, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia*

KIRK P. CONRAD, MD (2339), *Professor, Departments of Physiology and Functional Genomics, and of Obstetrics and Gynecology, University of Florida College of Medicine, Gainesville, Florida, USA*

STEVEN D. CROWLEY, MD (343), *Division of Nephrology, Duke University and Durham VA Medical Centers, Durham, North Carolina, USA*

NORMAN P. CURTHOYS, PhD (1601), *Professor Laureate, Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado, USA*

PEDRO R. CUTILLAS (979), *Head of Analytical Cell Signaling, Centre for Cell Signaling, Institute of Cancer, Barts and The London, Queen Mary's School of Medicine and Dentistry, Queen Mary, University College London, London, United Kingdom*

THEODORE M. DANOFF, MD, PhD (2477), *Group Director, Clinical Pharmacology and Discovery Medicine, Head, Human Target Validation Laboratory, Cardiovascular and Urogenital Center of Excellence, GlaxoSmithKline Pharmaceuticals, King of Prussia, Pennsylvania, USA*

EDWARD S. DEBNAM (979) *Department of Physiology, University College London, London, United Kingdom*

HENRIK DIMKE, MSc (1095), *The Water and Salt Research Center, Institute of Anatomy, University of Aarhus, Aarhus C, Denmark*

ALAIN DOUCET, PhD (57), *Laboratory of Renal Physiology and Genomics, Centre National de la Recherche Scientifique and Université Pierre et Marie Curie, Paris, France*

RAGHVENDRA K. DUBEY, MS, PhD (413), *Professor, Department of Obstetrics and Gynecology, Clinic for Reproduction-Endocrinology, University Hospital Zurich, Zurich, Switzerland and Associate Professor of Medicine, Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*

ADRIANA DUSSO, PhD (1803), *Renal Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA*

KAI-UWE ECKARDT, MD (2681), *Professor of Medicine, Chief, Department of Nephrology and Hypertension, University of Erlangen-Nuremberg, Erlangen, Germany*

DAVID H. ELLISON, MD (1051), *Head, Division of Nephrology and Hypertension, Oregon Health and Science University, Portland, Oregon, USA*

HITOSHI ENDOU, MD, PhD (185), *Professor Emeritus, Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan*

ZOLTÁN HUBA ENDRE (2507), *Professor, Head, Department of Medicine, University of Otago, Christchurch School of Medicine and Health Sciences, Christchurch, New Zealand*

FRANKLIN H. EPSTEIN, MD (1277), *William Applebaum Professor of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA*

ANDREW EVAN, PhD (1945), *Department of Anatomy and Cell Biology, Indiana University, Indianapolis, Indiana, USA*

RONALD J. FALK, MD (2315), *Division of Nephrology and Hypertension, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

KAMBIZ FARBAKHSH, MD (2591), *College of Medicine, University of Minnesota, Department of Medicine, Hennepin County Medical Center, Minneapolis, Minnesota, USA*

NICHOLAS R. FERRERI, PhD (359), *Department of Pharmacology, New York Medical College, Valhalla, New York, USA*

PEYING FONG, PhD (769), *Professor, Department of Anatomy and Physiology, Kansas State University, College of Veterinary Medicine, Manhattan, Kansas, USA*

MANASSES CLAUDINO FONTELES, PhD (463), *Reitoria, Universidade Presbiteriana Mackenzie, São Paulo, Brazil*

IAN FORSTER (1979), *Institute of Physiology, University of Zurich-Irchel, Zurich, Switzerland*

LEONARD RALPH FORTE, JR., PhD (463), *Department of Medical Pharmacology and Physiology, The Radiopharmaceutical Sciences Institute, University of Missouri School of Medicine, Senior Research Career Scientist, Truman Memorial VA Hospital, Columbia, Missouri, USA*

HAROLD A. FRANCH, MD (2615), *Renal Division, Emory University School of Medicine, Atlanta, Georgia, USA*

LYNDA A. FRASSETTO, MD (1621), *Associate Clinical Professor, Department of Medicine, University of California, San Francisco, San Francisco, California, USA*

PETER A. FRIEDMAN, PhD (1851), *Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennslyvania, USA*

JØRGEN FRØKIÆR, MD, DMSc (1095), *Professor, Chief Consultant, Department of Clinical Physiology, Aarhus University Hospital-Skejby, Aarhus, Denmark*

JOHN P. GEIBEL, MD, DSc (1269, 1785), *Department of Surgery and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

MICHAEL GEKLE, PhD (2021), *Julius-Bernstein-Institut für Physiologie, Martin-Luther-Universität Halle-Wittenberg, Halle, Germany*

GERHARD GIEBISCH, MD (1301*), Sterling Professor Emeritus Physiology, Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

PERE GINÈS, MD (2235), *Professor of Medicine, Chairman, Liver Unit, Hospital Clinic, University of Barcelona School of Medicine, Barcelona, Catalunya, Spain*

STEVE A.N. GOLDSTEIN, MD, PhD (1407), *Department of Pediatrics, The Institute for Molecular Pediatric Sciences and Comer Children's Hospital of the University of Chicago Pritzker School of Medicine, Chicago, Illinois, USA*

SIMIN GORAL, MD (2737), *Renal Electrolyte & Hypertension Division, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania, USA*

SIAN V. GRIFFIN, MD (723), *Consultant Nephrologist, University Hospital of Wales, Heath Park, Cardiff, Wales*

WILLIAM B. GUGGINO, PhD (769), *Professor and Director of Physiology, Professor of Pediatrics, Department of Physiology and Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA*

THERESA A. GUISE, MD (1911), *Gerald D. Aurbach Professor of Endocrinology, Professor of Medicine, Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia, Charlottesville, Virginia, USA*

SUSAN B. GURLEY, MD, PhD (343), *Division of Nephrology, Department of Medicine, Duke University and Durham VA Medical Centers, Durham, North Carolina, USA*

STEPHEN D. HALL, PhD (2763), *Professor of Medicine, Division of Clinical Pharmacology, Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana, USA*

MITCHELL L. HALPERIN, MD, FRCPC, FRS (1387), *Division of Nephrology, St. Michael's Hospital, Emeritus Professor of Medicine, University of Toronto, Toronto, Ontario, Canada*

L. LEE HAMM, MD (1539), *Huberwald Professor and Chair of Medicine***,** *Tulane University School of Medicine, New Orleans, Louisiana, USA*

STEVEN C. HEBERT, MD (1249, 1785), *C.N. H. Long Professor of Physiology and Medicine and Chair, Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

MATTHIAS A. HEDIGER, PhD (91), *Director, Institute for Biochemistry and Molecular Biology, University of Bern, Bern, Switzerland*

J. HAROLD HELDERMAN, MD (2737), *Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, Tennessee, USA*

WILLIAM L. HENRICH, MD, MACP (2193, 2719), *Dean, School of Medicine, Vice President for Medical Affairs, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA*

AILLEEN HERAS-HERZIG, MD (1911), *Assistant Professor of Research, Division of Endocrinology and Metabolism, University of Virginia, Charlottesville, Virginia, USA*

NATI HERNANDO (1979), *Institute of Physiology, University of Zurich-Irchel, Zurich, Switzerland*

JOOST G.J. HOENDEROP, PhD (1769), *Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*

ULLA HOLTBÄCK, MD, PhD (443), *Associate Professor, Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden*

JEAN-DANIEL HORISBERGER, MD (57), *Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland*

EDITH HUMMLER (889), *Department of Pharmacology and Toxicology, University of Lausanne School of Medicine, Lausanne, Switzerland*

TRACY E. HUNLEY, MD (385), *Department of Pediatrics, Division of Pediatric Nephrology, Monroe Carell Jr. Children's Hospital at Vanderbilt, Nashville, Tennessee, USA*

EDWIN K. JACKSON, PhD (413), *Professor of Pharmacology and Medicine, Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*

J. CHARLES JENNETTE, MD (2315), *Brinkhous Distinguished Professor and Chair of Pathology and Laboratory Medicine, Professor of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

EDWARD J. JOHNS (925), *Department of Physiology, University College Cork, Cork, Republic of Ireland*

JOHN P. JOHNSON (743), *Professor of Medicine and Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA*

BRIGITTE KAISSLING (479), *Institute for Anatomy, University of Zurich, Zurich, Switzerland*

KAMEL S. KAMEL, MD, FRCP (1387), *Professor of Medicine, Division of Nephrology, St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada*

S. ANANTH KARUMANCHI, MD (2339), *Associate Professor of Medicine, Beth Israel Deaconess Medical Center & Harvard Medical School, Boston, Massachusetts, USA*

CLIFFORD E. KASHTAN, MD (2447), *Professor of Pediatrics, University of Minnesota School of Medicine, University of Minnesota Children's Hospital-Fairview, Minneapolis, Minnesota, USA*

BERTRAM L. KASISKE, MD (2591), *Department of Medicine, University of Minnesota, Minneapolis, Minnesota, USA*

ADRIAN I. KATZ, MD (1349), *Professor Emeritus of Medicine, Section of Nephrology, University of Chicago, Chicago, Illinois, USA*

BRIAN F. KING (425), *Department of Physiology, University College London, London, United Kingdom*

SAULO KLAHR, MD (2247), *Professor Emeritus, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA*

THOMAS R. KLEYMAN, MD (743), *Chief, Renal-Electrolyte Division, Professor of Medicine, Cell Biology and Physiology, and Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

HERMANN KOEPSELL, MD (2045), *Professor of Anatomy and Cell Biology, Head of the Department, Institute of Anatomy and Cell Biology of the University of Würzburg, Würzburg, Germany*

VALENTINA KON, MD (385), *Associate Professor, Department of Pediatrics, Division of Pediatric Nephrology, Vanderbilt University School of Medicine, Memphis, Tennessee, USA*

MARTIN KONRAD, MD (1747), *Professor of Pediatric Nephrology, University Children's Hospital, Münster, Germany*

ULLA C. KOPP, PhD (925), *Department of Pharmacology, VA Medical Center, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA*

RETO KRAPF, MD (1667), *Professor of Medicine, Chief, Department of Medicine, Kantonsspital Bruderholz/University of Basel, Bruderholz/Basel, Switzerland*

WILHELM KRIZ (479), *Institute for Anatomy and Cell Biology, University of Heidelberg, Heidelberg, Germany*

RAJIV KUMAR, MBBS, MACP (1891, 1989), *Ruth and Vernon Taylor Professor, Departments of Internal Medicine, Biochemistry, and Molecular Biology, Mayo Clinic, Rochester, Minnesota, USA*

CHRISTINE E. KURSCHAT (1499), *Department of Nephrology, Internal Medicine IV, University Hospital of Cologne, Cologne, Germany*

ARMIN KURTZ, MD (2681), *Institut für Physiologie der Universität Regensburg, Regensburg, Germany*

TAE-HWAN KWON (1095) *Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea*

CHRISTOPHER P. LANDOWSKI (91), *Institute for Biochemistry and Molecular Biology, University of Bern, Bern, Switzerland*

ANTHONY J. LANGONE, MD (2737), *Division of Nephrology and Transplantation, Vanderbilt University Medical Center, Nashville, Tennessee, USA*

FLORIAN LANG, MD (169), *Department of Physiology, University of Tübingen Germany, Tübingen, Germany*

HAROLD E. LAYTON (1143), *Professor of Mathematics, Department of Mathematics, Duke University, Durham, North Carolina, USA*

THU H. LE, MD (343), *Assistant Professor, Division of Nephrology, Department of Medicine, Duke University and Durham VA Medical Centers, Durham, North Carolina, USA*

DANIEL I. LEVY, MD, PhD (1407), *Assistant Professor, Section of Nephrology, Department of Medicine, and The Institute for Molecular Pediatric Sciences, University of Chicago, Chicago, Illinois, USA*

SHIH-HUA LIN, MD (1387), *Professor of Medicine, National Defense Medical Center, Division of Nephrology, Tri-Service General Hospital, Taipei, Taiwan*

MARSHALL D. LINDHEIMER, MD (2339), *Professor (Emeritus) of Medicine, Obstetrics & Gynecology, and Clinical Pharmacology Biological Science Division, Pritzker School of Medicine, University of Chicago, Chicago, Illinois, USA*

CHRISTOPHER Y. LU, MD (2577), *Department of Internal Medicine, Nephrology, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA*

MICHAEL P. MADAIO, MD (2399), *Chief of Nephrology, Professor of Medicine, Temple University School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA*

NICOLAOS E. MADIAS, MD (1721), *Chairman, Department of Medicine, Caritas St. Elizabeth's Medical Center, Chief Academic Officer, Caritas Christi Health Care System, Maurice S. Segal, MD, Professor of Medicine, Tufts University School of Medicine, Boston, Massachusetts, USA*

GERHARD MALNIC (1301), *Professor of Physiology, Departamento de Fisiologie e Biofisica, Instituto de Ciencias, Universidade de São Paulo, São Paulo, Brazil*

KARL S. MATLIN, PhD (1), *Laboratory of Epithelial Pathobiology, Department of Surgery, Vontz Center for Molecular Studies, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA*

WILLIAM C. McCLELLAN (2615), *Renal Division, Emory University School of Medicine, Atlanta, Georgia, USA*

JOHN C. MCGIFF, MD (359), *Department of Pharmacology, New York Medical College, Valhalla, New York, USA*

C. CHARLES MICHEL (247), *Emeritus Professor of Physiology and Senior Research Investigator, Department of Bioengineering, Imperial College London, London, United Kingdom*

JEFFREY H. MINER, PhD (691), *Professor of Medicine, Renal Division and of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri, USA*

WILLIAM E. MITCH, MD (2615), *Gordon A. Cain Chair in Nephrology, Director, Division of Nephrology, Baylor College of Medicine, Houston, Texas, USA*

HIROKI MIYAZAKI, MD, PhD (185), *Institute of Biochemistry and Molecular Medicine, University of Bern, Bern, Switzerland*

ORSON W. MOE, MD (1645), *Professor, Department of Internal Medicine and Physiology, Charles and Jane Pak Center of Mineral Metabolism, University of Texas Southwestern Medical Center, Dallas, Texas, USA*

BRUCE A. MOLITORIS, MD (2143), *Professor of Medicine, Director of Nephrology, Director of The Indiana Center for Biological Microscopy, Indiana University School of Medicine, Indianapolis, Indiana, USA*

R. CURTIS MORRIS, JR., MD (1621), *Professor of Medicine, Pediatrics, and Radiology, University of California, San Francisco, San Francisco, California, USA*

SALIM K. MUJAIS, MD (1349), *Northbrook, Illinois, USA*

HEINI MURER, PhD (1979), *Institute of Physiology, Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland*

SHIGEAKI MUTO, MD, PhD (1301), *Associate Professor, Department of Nephrology, Jichi Medical School, Shimotsuke, Tochigi, Japan*

EUGENE NATTIE, MD (1587), *Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire, USA*

ERIC G. NEILSON, MD (2477), *Hugh Jackson Morgan Professor of Medicine and Cell and Developmental Biology Physician-In-Chief, Vanderbilt University Hospital; Chairman, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA*

SØREN NIELSEN, MD, PhD (1095), *Water and Salt Research Center, Institute of Anatomy, University of Aarhus, Aarhus, Denmark*

SANJAY K. NIGAM, MD (671), *Professor of Pediatrics, Department of Medicine and Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California, USA*

JOSEPH M. NOGUEIRA, MD (2193), *Assistant Professor of Medicine, Division of Nephrology, University of Maryland School Medical System, Baltimore, Maryland, USA*

MAN S. OH, MD (275), *Professor of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York, USA*

MARK D. OKUSA, MD (1051), *Division of Nephrology, University of Virginia Health System, Charlottesville, Virginia, USA*

TANYA M. OSICKA, PhD (2081), *Research Associate, Department of Medicine, University of Melbourne, Austin Health, Heidelberg, Victoria, Australia*

THOMAS L. PALLONE, MD (627), *Department of Medicine and Nephrology, University of Maryland at Baltimore, Baltimore, Maryland, USA*

BIFF F. PALMER, MD (1005, 2719), *Professor of Internal Medicine, Director Renal Fellowship Training Program, Department of Internal Medicine, Division of Nephrology, University of Texas Southwestern Medical Center, Dallas, Texas, USA*

LAWRENCE G. PALMER, PhD (211), *Professor of Physiology & Biophysics, Weill Medical College of Cornell University, New York, New York, USA*

MARK D. PARKER, PhD (1481), *Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA*

JOAN H. PARKS, MBA (1945), *Research Associate (Assistant Professor), Medicine,Department of Medicine, University of Chicago School of Medicine, Chicago, Illinois, USA*

PATRICIA A. PREISIG, PhD (1539), *Professor, Internal Medicine and Cellular and Molecular Physiology, Section of Nephrology, Yale University School of Medicine, New Haven, Connecticut, USA*

GARY A. QUAMME (1747), *Department of Medicine, Vancouver Hospital and Health Science Center Koerner Pavilion, University of British Columbia, Vancouver, British Columbia, Canada*

L. DARRYL QUARLES, MD (2671), *The Kidney Institute and Division of Nephrology, University of Kansas Medical Center, Kansas City, Kansas, USA*

RAYMOND QUIGLEY, MD (707), *Professor of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas, USA*

W. BRIAN REEVES, MD (849), *Chief of Nephrology and Professor of Medicine, Division of Nephrology, Penn State College of Medicine, Hershey, Pennsylvania, USA*

GIUSEPPE REMUZZI, MD (2563), *Director, Mario Negri Institute for Pharmacological Research Negri Bergamo Laboratories and Director, Division of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Bergamo, Italy*

LUIS REUSS, MD (35, 147), *Professor and Chair, Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, Texas, USA*

DANIELA RICCARDI, PhD (1785), *Reader, Physiology, Cardiff University, Cardiff, Wales*

BRIAN RINGHOFER, MD (2671), *The Kidney Institute and Division of Nephrology, University of Kansas Medical Center, Kansas City, Kansas, USA*

EBERHARD RITZ, MD (2537), *Department of Internal Medicine, Nierenzentrum, Ruperto Carola University, Heidelberg, Germany*

CHRISTOPHER J. RIVARD, PhD (1203), *Assistant Professor of Medicine, Division of Renal Diseases and Hypertension, University of Colorado Health Sciences Center, Denver, Colorado, USA*

GARY L. ROBERTSON, MD (1123), *Professor of Medicine Emeritus, Feinberg Medical School of Northwestern University, Chicago, Illinois, USA*

ROBERT M. ROSA, MD (1277), *Professor of Medicine, Executive Associate Dean for Clinical Affairs, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA*

BERNARD C. ROSSIER, MD (889), *Department of Pharmacology and Toxicology, University of Lausanne School of Medicine, Lausanne, Switzerland*

LEILEATA M. RUSSO, PhD (2081), *Program in Membrane Biology/Division of Nephrology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA*

HENRY SACKIN, PhD (211), *Rosalind Franklin University of Medicine and Science/The Chicago Medical School, North Chicago, Illinois, USA*

HIROYUKI SAKURAI, MD (671), *Associate Project Scientist, Division of Nephrology and Hypertension, Department of Medicine, University of California, San Diego, La Jolla, California, USA*

JEFF M. SANDS, MD (1143), *Juha P. Kokko Professor of Medicine and Physiology, Director, Renal Division, Associate Dean for Clinical and Translational Research, Emory University School of Medicine, Atlanta, Georgia, USA*

LISA M. SATLIN, MD (707), *Professor of Pediatric and Medicine, Mount Sinai School of Medicine, New York, New York, USA*

HEIDI SCHAEFER, MD (2737), *Division of Nephrology and Transplantation, Vanderbilt University Medical Center, Nashville, Tennessee, USA*

JEFFREY R. SCHELLING, MD (2399), *Associate Professor and Nephrology Division Chief, Department of Medicine, Metro Health Medical Center, Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio, USA*

LAURENT SCHILD (889), *Department of Pharmacology and Toxicology, University of Lausanne School of Medicine, Lausanne, Switzerland*

KARL P. SCHLINGMANN (1747), *Department of Pediatrics, Philipps-University, Marburg, Germany*

JÜRGEN B. SCHNERMANN, *MD (589), Chief, Kidney Disease Branch, National Institute of Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA*

ROBERT W. SCHRIER, MD (2235), *Professor of Medicine, University of Colorado School of Medicine, Denver, Colorado, USA*

ANTHONY SEBASTIAN, MD (1621), *Department of Medicine, Division of Nephrology, General Clinical Research Center, Special Projects Associate, Clinical and Translational Science, Institute (CTSI), CTSI Strategic Opportunities, Support Center, CTSI Clinical Research Center, University of California, San Francisco, San Francisco, California, USA*

JOHN R. SEDOR, MD (2399), *Kidney Disease Research Center, Rammelkamp Center for Research and Education, MetroHealth System, Case Western Reserve University, Cleveland, Ohio, USA*

YOAV SEGAL, MD, PhD (2447), *Assistant Professor of Medicine, University of Minnesota School of Medicine, Minneapolis VA Medical Center, Minneapolis, Minnesota, USA*

TAKASHI SEKINE, MD (185), *Associate Professor, Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*

DONALD W. SELDIN, MD (1005, 1645, 1667), *William Buchanan Professor of Internal Medicine, University of Texas Southwestern Medical School, Dallas, Texas, USA*

MARTIN SENITKO, MD (2577), *Nephrology Fellow, Department of Internal Medicine, Division of Nephrology, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA*

MALATHI SHAH, MD (2113), *Renal Fellow, Division of Nephrology/Hypertension, Northwestern University, Feinberg School of Medicine, Chicago, Illinois, USA*

SUDHIR V. SHAH, MD, FACP (2601), *Division of Nephrology, University of Arkansas School for Medical Sciences, Little Rock, Arkansas, USA*

STUART J. SHANKLAND, MD (723), *Professor of Medicine, Belding H. Scribner Endowed Chair in Medicine, Head, Division of Nephrology, Department of Medicine, University of Washington, Seattle, Washington, USA*

ASIF A. SHARFUDDIN, MD (2143), *Assistant Professor of Clinical Medicine, Division of Nephrology, Indiana University School of Medicine, Indianapolis, Indiana, USA*

KUMAR SHARMA, MD, F.A.H.A. (2215), *Professor of Medicine, Director of Translational Research in Kidney Disease, University of California at San Diego, La Jolla, California, USA*

SHAOHU SHENG, MD (743), *Research Assistant Professor, Department of Medicine, Renal-Electrolyte Division, University of Pittsburgh, Pittsburgh, Pennsylvania, USA*

DAVID G. SHIRLEY (425), *Centre for Nephrology, University College London, London, United Kingdom*

STEFAN SILBERNAGL, PhD (2021), *Department of Physiology, University of Würzburg, Würzburg, Germany*

STEPHEN M. SILVER, MD (1179), *Clinical Associate Professor of Medicine, University of Rochester School of Medicine and Dentistry, Head, Nephrology Unit, Rochester General Hospital, Rochester, New York, USA*

MEL SILVERMAN, MD, FRCP (C) (2007), *Professor of Medicine, University of Toronto and Division of Nephrology University Health Network, Toronto General Hospital, Toronto, Ontario, Canada*

EDUARDO SLATOPOLSKY, MD, FACP (1803), *Joseph Friedman Professor of renal Disease, Department of Medicine, Renal Division, Washington University School of Medicine, Physician Barnes-Jewish Hospital, St. Louis, Missouri, USA*

STEFAN SOMLO, MD, CNH (2283), *Long Professor, Departments of Internal Medicine and Genetics, Section of Nephrology, Yale University School of Medicine, New Haven, Connecticut, USA*

RICHARD H. STERNS, MD (1179), *Professor of Medicine, University of Rochester School of Medicine and Dentistry, Chief of Medicine, Rochester General Hospital, Rochester, New York, USA*

ANDREW K. STEWART (1499), *Department of Medicine, Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA*

YOSHIRO SUZUKI (91), *Institute for Biochemistry and Molecular Biology, University of Bern, Bern, Switzerland*

PETER J. TEBBEN, MD (1891), *Departments of Internal Medicine, Pediatrics, and Adolescent Medicine, Mayo Clinic, Rochester, Minnesota, USA*

SCOTT C. THOMSON, MD (565), *Professor of Medicine, Division of Nephrology-Hypertension, University of California and VA San Diego Healthcare System, San Diego, California, USA*

VICENTE E. TORRES, MD (2283), *Professor of Medicine, Chair, Division of Nephrology and Hypertension, Mayo Clinic College of Medicine, Rochester, Minnesota, USA*

ROBERT J. UNWIN, PhD, FRCP (425, 979), *Renal Physiology and Epithelial Transport Group, Centre for Nephrology and Department of Physiology, Royal Free and University College Medical School, University College London, London, United Kingdom*

FRANÇOIS VERREY (889), *Institute of Physiology, University of Zurich, Zurich, Switzerland*

DAVID L. VESELY, MD, PhD (947), *Professor of Medicine, Molecular Pharmacology and Physiology and Director of University of South Florida Cardiac Hormone Center, Chief of Endocrinology, Diabetes and Metabolism, James A. Haley Veterans Medical Center, Tampa, Florida, USA*

CARSTEN A. WAGNER (1269), *Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland*

MERYL WALDMAN, MD (2399), *National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA*

ROBERT JAMES WALKER (2507), *Head of Department, Department of Medical and Surgical Sciences, University of Otago, Dunedin School of Medicine, Dunedin, New Zealand*

WEI WANG, MD (1203), *Assistant Professor of Medicine, Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado, USA*

WEN-HUI WANG, MD (1249), *Professor of Pharmacology, Department of Pharmacology, New York Medical College, Valhalla, New York, USA*

YINGHONG WANG, MD PhD (769), *Department of Medicine, Albany Medical Center, Albany, New York, USA*

ALAN M. WEINSTEIN, MD (793), *Department of Physiology and Biophysics, Cornell University Weill Medical College, New York, New York, USA*

PAUL A. WELLING, MD (325), *Professor, Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland, USA*

GUNTER WOLF, MD (2215), *Professor of Medicine, Department of Internal Medicine, University Hospital Jena, Jena, Germany*

ELAINE WORCESTER, MD (1945), *Associate Professor of Medicine, Department of Medicine, University of Chicago School of Medicine, Chicago, Illinois, USA*

FUAD N. ZIYADEH, MD (2215), *Professor of Medicine & Biochemistry, Division of Nephrology, Department of Internal Medicine, Acting Chairman, Department of Physiology, Associate Dean for Academic Affairs-Faculty of Medicine, American University of Beirut, Beirut, Lebanon*

CARLA ZOJA, PhD (2563), *Mario Negri Institute for Pharmacological Research, Bergamo, Italy*

Foreword

The first edition of **The Kidney: Physiology and Pathophysiology** was published in 1985. Even at that early period, an abundance of books on kidney disease were in circulation. In general, the principal emphasis was on discrete renal disease, often presented as an isolated phenomenon, independent of the physiologic background generating the disease process. In part, this focus on the descriptive aspects of a disease was the consequence of the lack of basic understanding of the underlying pathophysiologic processes. In large measure, however, the analysis of abnormal kidney function in terms of discreet disease entities was also a conceptual model, derived largely from the triumphs in infectious disease, where a causal root to a disease could be unambiguously identified. The concept of abnormal function as an expression of deranged physiologic regulation was only dimly perceived.

The first edition took cognizance of this orientation and was designed to furnish a broad understanding of the regulatory function of the kidney as the basis for an analysis of renal abnormalities as derangements of regulation. The focus of the early edition was therefore mainly on renal physiology, "conceived broadly as the study of those processes by which the kidney maintains the volume and composition of the body in the face of physiologic demands and pathologic disturbances".

The second edition in 1992 and the third edition in 2000 greatly expanded the treatment of the basic processes underlying exchanges of water and electrolytes and their regulation by the kidney. The advances in structural and molecular biology, immunology, and genetics both deepened and broadened the analytic framework, allowing for a reduction of physiologic processes to very fundamental levels. At the same time the function of physiologic ensembles–the counter-current system, acid-base regulation, homeostatic balance–now characterized at a molecular level, could then be understood as an integrated interactive system.

This fourth edition is now in the editorial hands of two of our colleagues, Robert J. Alpern and Steven C. Hebert. Both are distinguished investigators. Dr. Alpern's principle interest has been the analysis of renal acidification, particularly the mechanisms in the proximal tubule cell which respond to changes in acid-base balance and maintain homeostasis–identifying genes participating in acid-base regulation, characterizing the behavior of transporters involved in acid secretion and bicarbonate efflux, and advancing the appreciation of the role of citrate in response to acid-base changes. These basic studies are accompanied by a deep competence in general renal physiology and clinical medicine that allows for a comprehensive appreciation of overall kidney physiology. His colleague Steven Hebert has made fundamental contributions to renal physiology, cloning transporters, characterizing channels and discovering a systems of calcium sensors in the kidney, parathyroid gland, and gastrointestinal tract which not only participate in the regulation of calcium and sodium balance but also function as a regulator of overall intestinal absorption and secretion and renal excretion, thereby acting as an internal homeostatic system. Dr. Hebert's interests range widely across the entire area of normal and deranged renal function.

In their hands, this fourth edition has taken on a fresh personality. There is increased emphasis on normal physiologic regulation and its disruption by disease; particularly noteworthy is the detailed attention to the specific mechanisms underlying pathologic changes. By deploying the most fundamental advances in renal physiology—not simply as isolated achievements, but as key ingredients to the understanding of physiologic regulation—this fourth edition constitutes a unique synthesis for the understanding of normal renal regulation and its derangement by disease.

Donald W. Seldin
Gerhard Giebisch

Preface

As described in its preface, the first edition of ***The Kidney: Physiology and Pathophysiology,*** published in 1985, focused on renal physiology, "conceived broadly as the study of those processes by which the kidney maintains the volume and composition of the body in the face of physiologic demands and pathologic disturbances." Since the publication of the first edition, science has become more reductionist, an evolution that has been reflected in the content of subsequent editions. Dissection of physiologic phenomena at the level of organs and cells was replaced by descriptions of the roles of individual molecules. As this trend in science has continued, so has the present edition continued to evolve in this direction. A complete understanding of physiologic processes must include knowledge of individual molecules—it should also include an integration of how these molecules work together to effect cellular and organ function that ultimately allow the system to address the requisite physiological demands.

The main focus of the **Fourth Edition** is to describe the present state of knowledge from molecules to systems that contribute to normal physiologic function of the kidney and the homeostatic mechanisms subserved by the kidney. The present edition will also concentrate on how these mechanisms malfunction resulting in the diseased state. Again we will address the pathophysiology of disease states from the molecular to the system level. One of the delightful features of nephrology is the ability to understand disease pathophysiology and to appreciate principles of clinical medicine. Thus, the clinician addressing a patient with a fluid and electrolyte disorder need not memorize a list of possible causes, but can deduce them through a thorough understanding of kidney function. As science continues to evolve, our understanding of the pathophysiologic basis of disease can now be applied to a much broader set of ailments. We, therefore, continue to broaden the scope of this book—to place greater emphasis on the mechanisms of disease.

Section One begins with general principles of epithelial and nonepithelial transport and regulation. This extensive section of the book continues a tradition established in the first edition, but builds on it to include a more extensive discussion of transport regulation. ***Section Two*** describes the organization of the kidney with an emphasis on renal development. ***Section Three*** follows, describing the mechanisms of fluid and electrolyte regulation and dysregulation. In no other book can one find this subject addressed with the depth and thoroughness found in this textbook. The **Fourth Edition** includes the most in-depth discussion of recently described families of transporters, integrating this information to describe their role in physiologic and pathophysiologic processes.

Section Four, the pathophysiology of renal disease, has been expanded as our knowledge of these processes and their contribution to renal ailments has grown. Of note is a new series of chapters focused on the *mechanisms of renal progression*. Progression of renal disease is a major area in which nephrologists can intervene to ensure that patients with asymptomatic increases in serum creatinine do not continue to lose kidney function, resulting in end stage renal disease. A thorough understanding of the roles of glomerular pressure, proteinuria, inflammation, lipids, and oxidants will allow researchers and clinicians to prevent renal failure, decreasing the need for dialysis and transplant.

The evolution of our understanding of kidney function and dysfunction derives from a series of discoveries made by a myriad of investigators, each benefiting from and building upon the accomplishments of their predecessors. The same can be said for textbooks. This textbook was originally conceived by the vision of two of the greatest renal physiologists of the twentieth century, Donald Seldin and Gerhard Giebisch. Their commitment to science and education created the vision for this book. It is our intent to continue their tradition and to honor them for all that they have contributed to this book, to nephrology, to epithelial physiology, and to science in general.

Robert J. Alpern, MD
Steven C. Hebert, MD

SECTION I

Epithelial and Nonepithelial Transport and Regulation

General Principles of Epithelial and Nonepithelial Transport

CHAPTER 1

Epithelial Cell Structure and Polarity

Karl S. Matlin and Michael J. Caplan
University of Cincinnati College of Medicine, Cincinnati, Ohio, USA
Yale University School of Medicine, New Haven, Connecticut, USA

INTRODUCTION

Many of the chapters in this volume are devoted to the mechanisms through which the nephron is able to convert the glomerular filtrate into a concentrated urine that is responsive to the metabolic status of the organism as a whole. The multifactorial nature of this problem necessitates that it be treated at several levels of resolution. A meaningful description of renal tubular function requires an understanding of the nephron's properties as an integrated tissue as well as those of its constituent parts, including the cells and molecules that contribute to its transport functions.

As is detailed elsewhere in this volume, the nephron is a remarkably heterogeneous structure. Throughout its length, the renal tubule is notable for the marked variations in the morphologic and physiologic properties of its epithelial cells, reflecting the numerous and diverse responsibilities that neighboring segments are called on to fulfill. At the tissue level, the function of the kidney is critically dependent on the geometry and topography of the nephron. The precise juxtaposition of various epithelial cell types, which manifest distinct fluid and electrolyte transport capabilities, in large measure specifies the course of modifications to which the glomerular filtrate is exposed. This dependence on geometry extends as well to renal function at the cellular level.

NATURE AND PHYSIOLOGIC IMPLICATIONS OF EPITHELIAL POLARITY

Despite their variations in form and function, all of the epithelial cells that line the nephron share at least one fundamental characteristic. Like their relatives in other epithelial tissues (including the intestine, lung, liver, etc.), all renal tubular epithelial cell types are polarized. The plasma membranes of polarized epithelial cells are divided into two morphologically and biochemically distinct domains (39, 184, 225, 269, 293). In the case of the nephron, the apical surfaces of the epithelial cells face the tubular lumen. The basolateral surface rests on the epithelial basement membrane and is in contact with the extracellular fluid compartment. The lipid and protein components of these two

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contiguous plasmalemmal domains are almost entirely dissimilar (39, 184, 225, 269, 293). It is precisely these differences that account for the epithelial cell's capacity to mediate the vectorial transport of solutes and fluid against steep concentration gradients. Thus, the subcellular geometry of renal epithelial cells is critical to renal function.

The principal cell of the collecting tubule provides a useful illustration of the importance of biochemical polarity for renal function. As described in other contributions to this volume, the principal cell is required to resorb sodium against a very steep concentration gradient. It accomplishes this task through the carefully controlled placement of ion pumps and channels (152, 245, 284). The basolateral plasma membrane of the principal cell, like that of most polarized epithelial cells, possesses a large complement of Na^+,K^+-ATPase. This basolateral sodium pump catalyzes the energetically unfavorable transport of three sodium ions out of the cell in exchange for two potassium ions through the consumption of the energy embodied in one molecule of ATP (310). The apical surface of the principal cell lacks a sodium pump, but is equipped with a sodium channel, which allows sodium ions to move passively down their concentration gradients (248). Through the action of the sodium pump the intracellular sodium concentration is kept low and the driving forces across the apical membrane favor the influx of sodium from the tubular fluid through the apical sodium channels. Thus, the combination of a basolateral Na^+,K^+-ATPase and an apical sodium channel lead to the vectorial movement of sodium from the tubule lumen to the interstitial space against its electrochemical gradient. This elegant mechanism is critically dependent on the principal cell's biochemical polarity. If the sodium pump and the sodium channel occupied the same plasmalemmal domain, then the gradients generated by the former could not be profitably exploited by the latter. Thus, the vectorial resorption or secretion of solutes or fluid is predicated on the asymmetric distribution of transport proteins in polarized epithelial cells.

The fact that epithelial cells manifest biochemical polarity implies that they are endowed with the capacity to generate and maintain differentiated subdomains of their cell surface membranes (39, 184, 225, 269, 293). Newly synthesized membrane proteins must be targeted to the appropriate cell surface domain and retained there following their delivery. During tissue development, cell division, and wound healing, plasmalemmal domains must be delimited and their biochemical character established. Clearly, specialized machinery and pathways must exist through which this energetically unfavorable compositional asymmetry can be supported. The nature of these specializations has been the subject of intense study for decades. While firm answers are not yet in, a number of fascinating model systems have been developed and valuable insights have emerged. This chapter will focus on what is known of the processes through which tubular epithelial cells create their polarized geometry.

EPITHELIAL CELL STRUCTURE: MORPHOLOGY AND PHYSIOLOGY

Morphologic Characteristics of Epithelial Polarity

As noted above (as well as in other contributions to this volume), the renal tubular epithelium is composed of a remarkably varied collection of cell types. A detailed delineation of its morphologic diversity is beyond the scope of this discussion. It may be valuable, however, to identify some of the salient structural features of certain renal epithelial subtypes, since they are illustrative of several more or less general aspects of epithelial organization.

Junctional Complex

All epithelial cells, including those of the kidney tubule, are joined together along the lateral surfaces by a series of intercellular junctions first noted by their characteristic ultrastructural appearances and relative locations on the lateral plasma membrane (72). These include the tight junction, or *zonula occludens*, the adherens junction (also known as the *zonula adherens* or intermediate junction), desmosomes, and gap junctions. In most epithelia, the tight junction is located at the apical-most edge of the lateral membrane closely followed by the adherens junction. Desmosomes and gap junctions have less specific locations on the lateral membrane.

Desmosomes are large multiprotein complexes responsible mainly for mechanical attachment between neighboring epithelial cells (286). By transmission electron microscopy they appear as discrete, focal concentrations of dense material in the cytoplasm of adjacent cells as well as in the intercellular space (72). They are composed of both integral membrane proteins of the cadherin family called desmogleins and desmocollins, and peripheral membrane proteins known as desmoplakins, as well as a variety of other protein constituents (286). Adjacent cells adhere to each other through cadherin-mediated interactions. The peripheral components then provide mechanical stability to this interaction via keratin intermediate filaments in the cytoplasm of each cell. Ultrastructurally, these appear as a mass of hair-like protrusions interacting in parallel with each plaque and then splaying out into the cytoplasm (72, 286). In this manner, desmosomes link all cells in the epithelium. While there is evidence that desmosomal components may play an active role in regulating some aspects of cell–cell adhesion and even gene expression (see below) (351), in general their function is considered to be relatively passive.

Gap junctions are so named because of the characteristic 3-nm gap that is evident by transmission electron microscopy between two interacting cells (291). Examination of freeze-fractured specimens reveals the gap junction as a discrete array of intramembranous particles or connexons (291). Each connexon is composed of five identical connexins, a family of transmembrane proteins. Connexons on adjacent cells interact through their extracytoplasmic domains to form a series

of low-resistance channels. These permit the passage of small molecules of less than 1 kD, both electrically and metabolically linking neighboring cells in the epithelium. In the kidney, it is likely that gap junctions play important roles during morphogenesis and repair, although their precise functions have not been investigated in detail (290).

Both tight junctions and adherens junctions are essential for establishing and maintaining polarization of epithelial cells, and for the correct physiologic functioning of the epithelium. For this reason, the structure and essential characteristics of each type of junction are extensively described in the next sections, followed by consideration of their roles in polarization.

Tight Junctions

Among the numerous functions subserved by epithelia, perhaps the most important is that of barrier between the intra- and extra-corporeal spaces. In the case of the kidney, the extracorporeal space is defined by the lumen of the renal tubule. The fact that the chemical composition of urine differs substantially from that of the extracellular fluid bathing the epithelial basolateral membranes is evidence that the barrier provided by the tubular epithelium is tight to small molecules. There are two components to this barrier, arranged in parallel (84). The first is comprised of the apical and basolateral plasma membranes of the epithelial cells themselves, which together serve as a pair of series resistances to the flow of solutes across the epithelia. The second barrier is provided by the intercellular junctions that join the epithelial cells to one another. The morphologic manifestation of this second component is the tight junctional ring, or zonula occludens (46, 99, 281, 303, 304).

The zonula occludens defines the border between the apical and basolateral plasma membrane surfaces. In columnar and cuboidal cells of the renal epithelium, it is found at the apical extremity of the lateral membrane and in the plane of the apical surface. Analysis by transmission electron microscopy suggested that the tight junction is actually a zone of partial fusion between the plasmalemmas of adjacent cells (72). When cells that have been treated with osmium are examined at high magnification, their membranes are distinguished by a characteristic pattern. The two leaflets of the lipid bilayer appear as a "unit membrane" defined by a pair of darkly staining parallel lines separated from one another by 5–10 nm (125). In areas corresponding to the tight junction, the four parallel lines representing the two unit membranes of the adjacent epithelial cells are replaced by three lines, which lead to the suggestion that the two outer leaflets contributed by the neighboring cells have in some way merged to form a new trilaminar membrane structure (75).

The putative outer-leaflet fusion suggested by morphologic studies received some support from examinations of lipid mobility in polarized epithelial cells. The mobility of outer-leaflet lipids is restricted by the tight junction (63, 327). Labeled lipid probes inserted into the outer leaflets of epithelial apical or basolateral plasmalemmas have unimpeded mobility within their respective domains but cannot cross the zonula occludens (63, 327). Furthermore, outer-leaflet lipids are unable to diffuse between neighboring epithelial cells through the tight junction. In contrast, inner-leaflet lipids can apparently move freely between the two plasma membrane domains, suggesting that the tight junction presents no barrier to their diffusion. These observations are consistent with a model of the zonula occludens in which the outer leaflets of the lipid bilayer participate in the formation of some junctional structure while the inner leaflet remains unperturbed. These results also suggest that the lipid composition of the apical inner leaflet is necessarily identical to that of the basolateral one, because any difference might be expected to be quickly randomized by diffusion. Thus, the differences in the lipid compositions of the apical and basolateral surfaces alluded to in the introductory paragraphs of this chapter must be entirely contributed by the constituents of the outer leaflet (328, 329).

Electron microscopy has provided further insights relevant to the structure of the zonula occludens. Examination of freeze-fracture replicas of epithelial cells reveals the tight junction to be composed of continuous branching and interwoven strands that surround the entire perimeter of the cell (299). These strands appear as elevations in the P, or cytoplasmic, fracture faces, and are matched by grooves in the E, or external, planes. The strands have a fibrillar appearance, and no discrete subunit structure can be resolved. It is now clear that these strands are composed of proteins known as claudins (90, 325). The claudin family includes more than a dozen members, each of which is a membrane protein that spans the membrane four times (210). Evidence that claudins comprise the principal structural components of the junctional strands derives from heterologous expression studies. Expression of claudins in fibroblast cells leads to the production of strands detectable by freeze fracture electron microscopy that closely resemble those associated with bona fide tight junctions in epithelial cells (87, 89). Claudins also determine the permeability properties of tight junctions (53, 88, 324, 326). In the kidney, the specific paracellular permeability characteristics found in each nephron segment are determined by the inventory of claudins expressed in their resident epithelial cells (292). Finally, it is interesting to note that the number and complexity of the strands seems to be correlated with the capacity of the junction to serve as a barrier (303). The number of parallel strands interposed between the apical and basolateral surfaces has been shown, in some systems, to be a rough indicator of the tightness of the junction as reflected in its electrical resistance.

In addition to the claudins, a large number of membrane and soluble proteins are associated with the zonula occludens (281, 304). The first protein to be identified in highly purified and extracted plasma membrane fractions is a polypeptide with a molecular weight of 225 kD named

ZO-1. In immunocytochemical experiments, antibodies raised against ZO-1 localize it exclusively to the tight junctional region of epithelial cells and to certain nonepithelial cells lacking tight junctions (303–305). Biochemical experiments reveal that ZO-1 is not a transmembrane protein, because it can be removed from plasmalemmal fractions by urea or alkaline extraction. ZO-1 is phosphorylated on serine residues, as well as tyrosine residues under certain circumstances, and is apparently released from the membrane under conditions in which intercellular tight junctions are disrupted (304). Sequencing of ZO-1 revealed that it belongs to a family of proteins known as MAGUK for membrane association and presence of the GUK domain. This family is characterized by the presence of one or more PDZ (PSD-95, discs large, ZO-1) domains, an src homology 3 (SH3) domain, and an area homologous to guanylate kinase (GUK), arranged sequentially on the molecule in the amino- to carboxy-terminal direction 280. Both PDZ and SH3 domains are involved in protein–protein interactions, and PDZ domains, in particular, may play important roles in basolateral localization and cell polarization (see later section). The GUK domain, which is not catalytically active in ZO-1, may also mediate protein–protein contacts. Recently, a splice variant of ZO-1 as well as two shorter homologues, ZO-2 and ZO-3 have been identified and sequenced (111, 120, 142, 304). In renal epithelial cells both ZO-2 and ZO-3 co-immunoprecipitate with ZO-1 and are localized exclusively to tight junctions. Biochemical studies suggest that both associate directly with ZO-1 but not with each other (111, 120, 142, 304). In subconfluent or injured epithelia, ZO-1 migrates to the nucleus, where it interacts with transcription factors to modulate the expression of genes involved in regulating growth control and differentiation (15, 16, 100).

The first transmembrane component of the tight junction identified was the protein occludin. Occludin is a phosphorylated polypeptide of 65 kD that is believed to span the membrane four times with both the amino- and carboxy-termini present on the cytoplasmic side (281, 304). There is in vitro evidence that occludin interacts with ZO-1. Because of its location in the membrane, occludin is believed to mediate cell–cell interactions via at least one of its extracellular loops. Indeed, treatment of cells with peptides corresponding to a loop sequence alters permeability and overexpression of occludin in fibroblasts increases adhesion (281, 304, 346). Recent studies using small interferring RNA technology to knock-down occludin indicate that it may facilitate signals to the actin cytoskeleton to help extrude apoptotic cells from the epithelium (346).

Nevertheless, occludin does not contribute to the interlocking strands of the tight junction because embryonic stem cells in which both occludin alleles have been deleted can still differentiate into epithelial aggregates with morphologically intact and physiologically functional tight junctions (276). In particular, ZO-1 localizes properly in these cells and the pattern of strands and grooves seen by electron microscopy of freeze-fractured specimens appears normal.

At least six other peripheral components of the tight junction have been identified (281, 304). Cingulin, which is homologous to cytoskeletal proteins bearing coiled-coil domains, has been localized further from the junctional membrane than ZO-1. Other notable proteins found at the junctions include small GTP-binding proteins in the rab family, as well as AF-6, which can bind ras, another small GTP-binding protein. Finally, actin, which is certainly not uniquely associated with the tight junction, has been reported to interact directly with ZO-1 in nonepithelial cells. As mentioned earlier, there is evidence that contraction of actin in the terminal web of epithelial cells can substantially alter transepithelial permeability (177, 235, 322).

Adherens Junctions

The adherens junction, or zonula adherens, forms a belt just below the tight junction in most epithelial cells connecting them via extracellular interactions and cytoplasmic linkages to the actin cytoskeleton. In the electron microscope, adherens junctions appear as a dense, somewhat amorphous concentration of submembranous staining, with a mass of impinging actin filaments (72). The major adhesive component of the adherens junction in epithelial cells is E-cadherin (originally called uvomorulin) (343). E-cadherin is a single-pass transmembrane protein that consists of a series of calcium-binding extracellular or EC domains, and a cytoplasmic tail that interacts with a protein called β-catenin. In the membrane, E-cadherin exists as a homodimer, and, while concentrated in the adherens junction, may be distributed over the entire basolateral membrane. β-catenin is homologous to the protein plakoglobin (or α-catenin), that is found mainly in desmosomes but sometimes substitutes for β-catenin in adherens junctions (see below) (343). Both E-cadherin and β-catenin are linked to the actin cytoskeleton through α-catenin, which binds to β-catenin, and is also found to a lesser extent in both tight junctions and desmosomes. In addition to the catenins, there is evidence that a number of other proteins involved in signaling also associate with E-cadherin (343).

In the presence of calcium, epithelial cells adhere to each other initially via E-cadherin. These interactions trigger a number of other events in the cell, some of which are only now beginning to be understood. Formation of tight junctions, for example, is dependent on E-cadherin–mediated linkages, as is the establishment of desmosomes. Cultured epithelial cell lines, for example, will attach to the culture substratum in the near absence of calcium. Under these conditions, not only do adherens junctions fail to form, but neither do tight junctions or desmosomes. As soon as normal concentrations of calcium are added back to the medium, at least adherens junctions and tight junctions rapidly assemble, as demonstrated by the detection of transmonolayer electrical resistance within minutes (231, 330). This

hierarchical relationship may be mainly mechanical, with E-cadherin interactions pulling the membranes sufficiently close together to enable the other junctions to form. Alternatively, it is possible that cytoplasmic signals generated by E-cadherin–dependent adhesion somehow activates or initiates assembly of the other junctions.

While the extracellular domain of E-cadherin is intrinsically adhesive, formation of fully functional junctions depends on the cytoplasmic domain and its interaction with the catenins and actin (343). This has been demonstrated in reconstruction experiments in which E-cadherin is expressed in nonepithelial cells (196, 222, 223, 247). While these do not normally form adherens junctions, they still express catenins. Thus, in the presence of exogenous E-cadherin they will adhere to one another, forming a monolayer whose appearance resembles a true epithelium by light microscopy. When E-cadherin mutants lacking the cytoplasmic tail are expressed, some cell–cell adhesion is detected, but it is mechanically unstable and there is no colocalization of catenins or concentration of actin in the region of cell–cell contacts.

In an interesting twist, expression of a chimeric form of E-cadherin fused to α-catenin obviates the need for α-catenin, leading to the formation of fully developed adherens junctions (221). Based on this experiment, one might ask why β-catenin is needed at all as an adapter between E-cadherin and α-catenin. The answer is apparently that β-catenin is an important regulatory molecule of both cell adhesion and gene expression in epithelial cells (343). This conclusion was reached through an amazing confluence of lines of investigation in both cell and cancer biology. Studies of transformed epithelial cells over a number of years demonstrated that transformation in general and invasive behavior in particular seemed to correlate with loss of E-cadherin (17, 20, 112, 351). Originally, this was explained mechanically; clearly, for cells to migrate during invasion of other tissues, cell–cell contacts had to break. Perhaps transformation led to the downregulation of E-cadherin as well as other components of the adherens junction. Independently, other investigators studying the genetics of colon cancer identified a gene for familial adenomatous polyposis coli (APC), which leads to a high frequency of colonic polyps and early incidence of colon cancer (17, 112, 351). The gene product of this APC gene turned out to be a cytoplasmic protein that binds β-catenin and facilitates its proteolytic destruction. In the absence of functional APC, β-catenin not bound to E-cadherin enters the nucleus where it is capable of activating certain genes contributing to carcinogenesis. Recently, it has been found that the degradation of β-catenin captured by the APC protein is negatively regulated by the Wnt/Frz pathway, the mammalian analogue of the wingless/frizzled/disheveled pathway originally described in *Drosophila* (17, 112, 351). When Wnt binds its receptor Frz in the plasma membrane it activates dsh. This in turn inhibits glycogen synthase kinase 3β (GSK3β), a component of the APC-β-catenin complex, preventing its phosphorylation of β-catenin and subsequent degradation. In this manner, the available APC protein in the cell becomes saturated with β-catenin, and excess free-cytoplasmic β-catenin enters the nucleus. Thus, β-catenin has three possible fates in the cell. It can bind E-cadherin and facilitate cytoskeletal association with the adherens junction. Alternatively, it can float free in the cytoplasm where it will either bind the APC protein and be proteolytically degraded, or enter the nucleus and activate gene expression (17, 112, 351). As complex as this regulatory pathway seems, the description provided here is undoubtedly oversimplified. APC protein also has a binding site for other proteins, including tubulin and axin (17). Furthermore, how transformation regulates the amount of E-cadherin on the cell surface, one of the original observations that led to the discovery of β-catenin's regulatory role, remains unclear. One possibility is that nuclear β-catenin can affect the expression of E-cadherin, although this remains unproven (17, 112, 351). As will be described in the subsequent section, E-cadherin plays an essential role in epithelial cell polarization. Thus, β-catenin regulation is key to understanding the organization of epithelia.

Apical Microvillar Surface

The apical brush border membrane is perhaps best epitomized by the one that graces the epithelial cells of the proximal tubule. Named for its appearance, the proximal tubular brush border is comprised of densely packed parallel microvilli that rise like the bristles of a brush from the level of the tight junctions to a height of 1 to 1.3 μm. The proximal tubular brush border is far and away the most luxuriant to be found in the nephron; although the apical membranes of other renal epithelial cell types are endowed with small collections of microvilli, much less is known about the structural specializations characteristic of the apical membranes of more distal renal epithelial cells (107).

The functions subserved by apical microvilli are not entirely clear. Certainly their most dramatic and obvious effect on the properties of the apical membrane is manifest as a tremendous amplification of the apical membrane surface area. For the proximal tubule this amplification is on the order of 20-fold (193, 340). As is the case for the epithelia of the small intestine, it is through this redundancy that the proximal tubular epithelial cells markedly increase the efficiency of both their absorptive and degradative functions.

Physiologically, the proximal tubule is responsible for the resorption of ~60% of the filtered load of fluid and solutes (179). Furthermore, it mediates the digestion of essentially all of the polysaccharides and peptides present in the glomerular filtrate, and transports the resultant sugars and amino acids from the lumen to the interstitial fluid space (193). It is apparent, therefore, that the epithelial cells of the proximal tubule must be specially equipped in order to cope efficiently with the comparatively enormous quantities of fluid and substrates that rapidly transit this nephron

segment. The presence of an extravagant brush border greatly increases the fraction of the tubular fluid that comes into close contact with the enzymatic and transport systems arrayed on the microvillar surfaces prior to its passage from this tubule segment into the descending loop of Henle. Concomitantly, it proportionally multiplies the number of enzymatic and transport systems available to modify the substrates dissolved in the tubular fluid. Thus, the brush border membrane provides the scaffolding for the relatively massive arsenal of enzymatic and transport machinery required to accomplish the proximal tubule's function as a high capacity and high throughput resorptive system.

Ultrastructurally, a microvillus is composed of a bundle of 20–30 parallel thin filaments that are linked to one another and to the overlying surface membrane by protein cross-bridges (207). The thin filaments extend well beyond the base of the microvillus and are anchored in a dense matrix of fibers oriented parallel to the plane of the membrane. This meshwork, referred to as the terminal web, underlies the entire apical surface and anastomses with the filaments that radiate from the lateral desmosomes and zonulae adherens. The functional implications of these structural arrangements have become clearer as their components have been biochemically identified.

The thin filaments that form the microvillar core are composed of actin (31, 207). Ultrastructural studies employing heavy meromyosin reveal that all of the filaments in the bundle share a single polarity and are oriented with their nucleating end towards the microvillar tip. At their termination in the microvillar tip the filaments are received by an electron-dense cap whose molecular identity has yet to be established (207). As they emerge from the base of the microvillus, the actin filaments are caught up in the fibers of the terminal web. Fodrin, or nonerythroid spectrin, comprises one of the major components of this network (97, 207). It appears to function beneath the brush border as an actin fiber cross-linker. Another of the chief constituents of this fibrillar matrix is a nonmuscle form of myosin II that belongs to the same myosin subfamily as its skeletal muscle counterpart. Bipolar myosin thick filaments appear to interact with the actin filaments as they sweep out of the microvillar sheath to join the terminal web (32, 65, 207). Paired antiparallel myosin filaments cross-link the actin filaments of neighboring microvilli to one another, forming a connection that bears close comparison to the actin–myosin arrangement characteristic of the striated muscle sarcomere. The analogy is strengthened by the presence in the microvillar rootlet of tropomyosin, a protein that functions in skeletal muscle to regulate the interaction between actin and myosin (65, 128).

This marked molecular similarity between the terminal web and the skeletal muscle contractile unit prompted speculation that this arrangement might also be functionally homologous. A number of investigators have postulated that activation of myosin-based contraction at the microvillar base might lead to microvillar shortening (206). Repetitive activation of such a mechanism would lead to a piston-like extension and retraction of these membranous processes, which in turn might stir the surrounding tubular fluid. Such a mixing motion is certainly teleologically appealing, in that it would help to ensure that the tubular fluid is uniformly exposed to the enzymatic and transport systems of the proximal tubular apical membrane surface. No evidence for any such concerted and dynamic properties of microvilli has yet been gathered.

Biochemical studies have shed light on the identities and functional properties of some of the proteins that contribute to the interfibrillar cross-bridges observed in transmission electron micrographic profiles of microvilli. Howe and Mooseker (131) identified a protein of molecular weight 110 kDa that participates in cross-linking the filaments of intestinal microvilli to the plasma membrane. This protein exhibits a high affinity for the calcium-binding protein calmodulin, which participates in the transduction of a number of calcium-regulated phenomena (131). Of further interest was the fact that the 110-kDa protein manifests a myosin-like Mg-ATPase activity (209). Addition of ATP to intact microvilli results in solubilization of the 110-kDa protein and disruption of the cross-links between the actin filaments and the microvillar membrane (56, 188). Thus, attachment of the plasma membrane to the thin filaments may be regulated by ATP and calcium. The degree to which this putative capacity for structural modulation plays a role in microvillar function has yet to be clarified. Subsequent molecular analysis revealed that the brush border 110-kDa protein belongs to the myosin I family of unconventional myosin molecules (92, 130). Unlike skeletal muscle myosin (which is assigned to the myosin II classification), brush border myosin I molecules possess a single globular head group and do not form bipolar filaments (49, 55, 208, 209). Members of the myosin I family, including brush border myosin I, have been found to associate with the membranes of intracellular vesicles, prompting the hypothesis that these motor proteins serve to propel vesicles through the cytoplasm along actin filament tracks (64). Co-localization studies have demonstrated that brush border myosin I and the microtubule-dependent motor protein dynein can be found together on the membranes of post-Golgi vesicles (73). This observation has inspired the hypothesis that apically directed vesicles depart the Golgi along microtubule tracks powered by the action of dynein. Upon their arrival at the actin-rich terminal web, they switch engines and are carried the rest of the way to the brush border by myosin I (74). While brush border myosin I is abundantly expressed in intestinal epithelial cells, it may be present at lower levels in the renal proximal tubule (19). Since the myosin I family is large and diverse, however, it is extremely likely that an as yet unidentified member of this class subserves similar structural and mechanical functions in the epithelial cells of the kidney (58).

Another protein that apparently participates in the organization of the microvillus has a molecular weight of 95 kDa

and has been dubbed *villin* (33). A cDNA encoding the villin molecule has been isolated and sequenced (9). It is apparent from this analysis that villin belongs to a large family of actin-binding proteins. Prominent in its structure is a pair of sequence domains that appear to be involved in associations with f-actin. The presence of this tandem repeat justifies the contention that villin mediates the bundling of actin fibers. It is interesting to note that villin is a calcium-binding protein and that interaction with calcium alters its behavior in the presence of actin filaments (189). In experiments carried out with purified villin in solution, it has been found that this protein bundles actin filaments when the free calcium concentration is less than 1 μM. When the calcium concentration rises to 10 μM, villin severs actin filaments into short protofilaments. At intermediate calcium concentrations, villin binds to actin filaments at their growing ends, forming a cap that prevents further elongation. Due to the dynamic nature of the microfilament polymer, this capping results in the formation of shortened filaments. It is not known whether these calcium-dependent activities of villin are manifest in vivo. If villin does indeed sever or shorten actin filaments within the living cell, it would seem likely that perturbations which produce elevations of intracellular calcium concentrations may lead to structurally significant alterations in the organization of the microvillar scaffolding. During embryonic development, villin is expressed throughout the cytoplasm of epithelial cells prior to the elevation of a brush border (265). At later stages, villin becomes localized to the cytosolic surface of the apical membrane and is subsequently incorporated into forming microvilli. This behavior has led to the suggestion that the localization of villin to the apical surface is a watershed event in the biogenesis of microvilli. Interestingly, expression of the cDNA encoding villin in fibroblasts, which normally lack microvillar processes, results in the formation of microvillus-like structures (83). Thus, the formation of interfilamentous bridges presumably mediated by villin may be a critical first step in the organization of the microvillar infrastructure. Supporting this model are the results of experiments in which Caco-2 intestinal epithelial cells were stably transfected with a vector encoding antisense villin mRNA (57). The consequent reduction in villin expression resulted in a loss of the brush border and mis-sorting of a subset of apical microvillar proteins. It must be noted, however, that results from gene knockout experiments argue against a central role for villin in microvillus formation (255). Mice whose villin genes have been disrupted and which produce no villin protein are able nonetheless to generate morphologically and apparently physiologically normal brush borders. Presumably, other components of the microvillar infrastructure can shoulder the cross-linking and organizational duties normally performed by villin. Such functional redundancy is typical of biological systems endowed with architecture as esthetically elegant and complex as that which graces the microvillus.

While villin is limited in its distribution to those cell types endowed with brush borders, another actin-bundling component of the microvillus is present in numerous structures. Fimbrin is a 68-kDa polypeptide associated with the interfilamentous cross-bridges that can also be detected in hair cell stereocilia and in ruffled borders (31). Fimbrin is clearly a multivalent actin-binding protein and participates in the cross-linking of the microvillar actin filament array. The degree to which its role in this process is related to or distinct from that of villin has yet to be established. Several other polypeptides associated with the microvillar core have also been identified. A protein of molecular weight 80 kDa that exhibits homology with a substrate of the epidermal growth factor receptor tyrosine kinase suggests another possible pathway through which microvillar structure and function might be manipulated (104). A 200-kDa protein has been identified which may serve as the transmembrane anchor for the 110-kDa myosin I–like protein discussed above (59). This protein was isolated from porcine intestinal microvilli and may be cleaved to a 140-kDa form during development. In vitro studies suggest that this glycoprotein manifests an affinity for the 110-kDa myosin I polypeptide. It should be noted, however, that studies suggest that the myosin I protein can interact with high affinity with protein-free liposomes composed of negatively charged phospholipids (122). These observations suggest the possibility that myosin I might link actin filaments directly to the lipids of the overlying plasmalemma without any requirement for a transmembrane proteinaceous adapter. Arguing against this possibility are recent results demonstrating that although phospholipid-bound myosin I is active as an ATPase, when attached to membranes in this configuration it loses its capacity to serve as an actin-based motor (354). The nature of all of these interactions remains to be elucidated. Several other proteins have been identified as possible links between microvillar actin filaments and the overlying plasma membrane. Zipper protein is a transmembrane polypeptide that derives its name from a cluster of 27 leucine zipper heptad repeats (25). The C-terminal domain of zipper protein can compete with tropomyosin for binding to actin filaments, suggesting that both polypeptides interact with the same binding site. Zipper protein can also inhibit actin activation of the brush-border myosin I ATPase activity, although it has no effect on the myosin's endogenous ATPase activity. These observations are consistent with the possibility that zipper protein may regulate the association of microvillar actin filaments with molecular motors and other mechanotransducing proteins. Finally, a similar linking function has been ascribed to members of the ezrin-radixin-moesin family of proteins (30). The C-terminal tails of these polypeptides bind to actin filaments, while their N-termini interact with proteins in the membrane. It has also been shown that a number of proteins involved in the generation or regulation of intracellular second messengers associate in macromolecular complexes with ezrin-radixin-moesin family members, suggesting that in

addition to functioning as linkers these proteins may also act as scaffolding for the assembly of components involved in signal transduction.

The terminal web mentioned above consists of three morphologically distinguishable domains. In addition to the cytoskeletal fibers that receive the rootlets of the microvilli, fibers that arise from desmosomes and the zonula adherens contribute to this meshwork. The desmosomal fibers consist primarily of 10-nm intermediate filaments composed of keratins (81). At the level of the zonula adherens, the cell is ringed by a complex of randomly polarized actin filaments that also contains myosin and tropomyosin (65). In vitro experiments have demonstrated that this ring has the capacity to contract circumferentially (38). This capacity has led to the speculation that contraction of the zonula adherens ring might contribute to the alterations in tight junctional permeability that have been observed in several epithelial systems in response to certain second messengers and osmotic stress (177). Thus, activation of sodium-coupled glucose uptake in cultured intestinal epithelial cells has been shown to induce a decrease in transepithelial resistance. This effect is dependent on the activity of myosin light-chain kinase (123, 322). It is thought that by shortening in a "purse-string" fashion, these filaments might actually draw neighboring cells away from one another and thus modify the structure and permeability of the occluding junctions. The relevance of this model to the functioning of renal epithelia has yet to be established.

The anisotropy and structural complexity that characterize the filamentous core of the microvillus apparently extend as well to its overlying plasma membrane. The proteins embedded in and associated with the plasmalemma of the proximal tubule brush border are not uniformly distributed over its surface but rather are restricted to specific subdomains. This lateral segregation is epitomized by the behavior of two transmembrane polypeptides, maltase and gp330. The 300-kDa enzyme maltase is distributed over the entire surface of the microvilli themselves, but is absent from the intermicrovillar membrane regions (146, 266). In contrast, the heavily glycosylated gp330 (also known as megalin) is restricted in its distribution to these intermicrovillar regions. The restriction of megalin to the intermicrovillar regions appears to be mediated by its interactions with protein components of the endocytic machinery (224). Ultrastructural examination of the intermicrovillar regions reveals the presence of coated pits. The cytosolic surface of the plasma membrane in these domains is coated with an electron-dense material that biochemical and immunoelectron microscopic studies have demonstrated to be clathrin (266). The presence in these intermicrovillar pits of morphologic and compositional features associated with the process of endocytosis has led investigators to believe that this domain mediates the retrieval of large peptides and proteins from the proximal tubular fluid. The proximal tubular epithelial cells are responsible for capturing and degrading any proteins that pass through the glomerular filtration barrier (193). This function is apparently served by the profusion of coated pits and vesicles that decorate the surfaces of membranes at the microvillar base. The function of gp330/megalin has recently been clearly elucidated. Megalin is a member of the low density lipoprotein (LDL) receptor family and, together with cubulin, serves as receptor that binds to and mediates the uptake of filtered proteins and peptides (27). Megalin knockout mice exhibit low-molecular-weight proteinuria, establishing the critical role for megalin as the proximal tubule's preeminent scavenger (164, 236).

Finally, it is worth noting that most or all of the epithelial cells of the nephron are endowed with a single primary cilium (256). This nonmotile cilium possesses an outer ring of nine microtubules but lacks the central pair of microtubules found in motile cilia. This primary cilium appears to serve sensory functions. Bending the primary cilium, in response to flow or mechanical stimuli, induces calcium signaling in renal epithelial cells (257). Furthermore, the functional integrity of the primary cilium appears to be a prerequisite for the maintenance of normal renal tubular architecture. A number of cystic diseases of the kidney are attributable to mutations in genes encoding proteins found in cilia (170, 172, 205, 345). Similarly, mice in which expression of ciliary proteins has been disrupted develop cysts. The mechanism through which loss of the cilium's mechanosensory functions leads to cystic transformation remains to be established.

Basolateral Plasma Membrane

The rigid subservience of structure to function so elegantly exemplified by the apical brush-border membrane extends as well to the basolateral surface of the epithelial plasma membrane. As was mentioned above, the basolateral membrane possesses the ion pumps that power the transepithelial resorption of solutes and water. The resorptive capacity of a given cell type is thus largely dependent on the quantity of ion pumps embedded within its basolateral plasmalemma. This parameter appears in turn to be roughly proportional to the surface area encompassed by this membrane domain (245). Consequently, renal epithelial cells that participate in the resorption of large quantities of ions and fluid (such as those of the proximal tubule) as well as cells that carry out resorption of ions against steep concentration gradients (such as those of the thick ascending limb of the loop of Henle) are endowed with basolateral plasmalemmas whose surface areas are amplified through massively redundant infoldings.

As was detailed in the discussion of the apical brush border, the lateral distribution of proteins within the plane of the basolateral membrane is not uniform. This fact is most dramatically illustrated by epithelial cell types that lack the deeply invaginated basolateral infoldings discussed above. Studies have demonstrated that the Na^+,K^+-ATPase is concentrated in subdomains of the basolateral membranes of small intestinal epithelial cells (6). The sodium pump is essentially restricted to the lateral membranes of

these cells and is absent from the basal surfaces that rest on the basement membrane. Dislodging these cells from the underlying basement membrane produces a redistribution of the sodium pump throughout the entire basolateral plasmalemma. These results suggest that the sodium pump is either actively or passively prevented from entering the basal domain of the plasmalemma in some manner that is dependent on an intact interaction with the basement membrane. The meshwork of cytoskeletal elements associated with those sites at which the epithelial cell is anchored to basement membrane fibrils may be too dense to allow membrane proteins such as the sodium pump to penetrate. Conversely, cytoskeletal restraints whose integrity requires cell attachment to the basement membrane might retain the sodium pump within the lateral subdomains. In each of these scenarios, the cytoskeleton plays an important role in determining the subcellular distribution of a transmembrane protein. Recent research has made it quite clear that the cytoskeleton plays a critical role in defining polarized domains and in determining aspects of their polypeptide compositions (2, 196, 211, 226–229, 231, 249, 264, 277, 336). The role of the cytoskeleton in the generation and maintenance of polarized distributions of membrane proteins will be discussed later in this chapter.

Polarized Distribution of Organelles

The massive complement of ATP driven ion pumps deployed in the basolateral infoldings consumes a significant fraction of the epithelial cell's metabolic energy. It is not surprising, therefore, that the cytosolic spaces between adjacent infoldings are frequently occupied by mitochondria. In the proximal tubule, these mitochondria are oriented parallel to the infoldings. This vertical alignment of the mitochondria associated with the basolateral surface gives rise to this membrane domain's typically striated appearance when examined by light microscopy (318). The mechanism through which mitochondria come to be located in the regions of the cell that are precisely engineered to use energy is entirely unknown. Presumably, this localization is brought about through interactions between the mitochondria and elements of the cytoskeleton such as microtubules. Precedent for mitochondrial–microtubular interaction exists in elegant experiments performed on neuronal axoplasm that demonstrate that mitochondria "crawl" on microtubule tracks with the help of the ATP-driven kinesin motor (323). It is clear that this juxtaposition ensures that energy is delivered to the transport enzymes of the basolateral surface with the smallest possible diffusional losses.

This discussion highlights the fact that the term "polarized," as applied to epithelia, refers not only to the distribution of plasmalemmal proteins, but also to the arrangement of cytosolic structures and organelles. In addition to the nonrandom distribution of mitochondria, epithelial cells are notable for apically disposed Golgi complexes as well as basally positioned nuclei and endoplasmic reticulum (ER) (72). Specialized, cell-type specific structures are also distributed with polarity. Thus, the apical cytoplasm of α-intercalated cells is populated by acidic endocytic vesicles (285). In contrast, the acidic endosomal vesicles of β-intercalated cells are restricted to cytoplasm in the vicinity of the basolateral plasma membrane (285). Finally, in the absence of antidiuretic hormone stimulation the principal cells of the collecting tubule store transmembrane water channels in vesicles that gather in the apical cytoplasm (118, 167, 333). These examples support the concept that the anisotropy characteristic of epithelia extends to every aspect of their organization. Clearly, epithelial cells organize themselves, both at the molecular and organellar levels, along an axis determined by external stimuli (225, 269). As discussed later in this chapter, it is currently thought that the most important of these external stimuli is contact with the epithelial basement membrane and with adjacent epithelial cells (225, 231, 269, 272, 330, 331, 336). The machinery that transduces and responds to these stimuli includes the integrin family of basement membrane receptors, cell adhesion molecules, and elements of the cytoskeleton with which these families of molecules interact.

BIOGENESIS OF EPITHELIAL POLARITY

In Vitro Systems

The kidney's complicated architecture and cellular heterogeneity renders it a poor substrate for studies designed to examine dynamic cell biologic processes. Over the past three decades, the vast majority of research into the mechanisms through which epithelia generate and maintain their polarized phenotype has made use of several continuous lines of cultured epithelial cells. These cell lines retain many of the differentiated properties of their respective parent tissues in vitro. Thus, LLC-PK1 cells resemble the proximal tubule (although their precise origin is uncertain) (293). Similarly, Caco-2, HT-29, and T84 cells behave like their progenitors, the colonocytes of the large intestine (293). Most importantly for the purposes of this discussion, they manifest in culture the biochemical and morphologic features of the polarized state. Perhaps the best characterized and most heavily used of these culture models is the Madin–Darby canine kidney (MDCK) line. MDCK cells were originally derived from a normal dog kidney in 1959 and grown in culture as a partially transformed line; that is, MDCK cells grow immortally as a monolayer and will not form tumors in nude mice (93, 178). Although their precise point of origin along the nephron is not entirely clear, their physiologic and morphologic properties suggest that they derive from cells of the thick ascending limb, distal tubule, or collecting tubule (127).

The first clues to the polarized nature of the MDCK cell line came from the direct observation of these cells' capacity for vectorial transport. When grown on impermeable

substrata, MDCK cells form domes (also called blisters or hemicysts) (165). Physiologic studies have demonstrated that domes develop as a result of the transepithelial transport of solutes from the apical media to the basolateral surface (1). Water that passively follows these solutes results in the generation of fluid-filled blisters. It is fair to say that domes arise in regions where the cells have literally pumped themselves up off the dish. In keeping with this dramatic propensity for unidirectional solute movement, each MDCK cell manifests a polarized distribution of ion-transport proteins, including several routes for sodium entry in its apical membrane and of the order of 10^6 molecules of the Na^+,K^+-ATPase in its basolateral plasmalemma (45). The popularity of MDCK cells for polarity research developed out of the seminal observations of Rodriquez-Boulan and Sabatini in 1978 (268). In studies on the budding of enveloped viruses from MDCK cells, these investigators found that the influenza virus assembles at, and buds from, the apical cell surface (Fig. 1). Of even greater significance was the demonstration that the spike glycoproteins which populate the membranes of these viruses accumulate preferentially at the cell surface from which budding is to occur (267). Thus, the influenza hemagglutinin (HA) protein is predominately on the apical surface of MDCK cells early in infection. Similarly, the G protein of vesicular stomatitis virus (VSV) is almost exclusively basolateral in infected cells. The viral proteins provided investigators with the first experimentally manipulable system for the study of membrane protein sorting. A large number of studies have subsequently elucidated the sorting of many endogenous MDCK cell proteins as well as exogenous proteins expressed from vectors. This system remains the most thoroughly investigated paradigm and, as will be detailed below, has yielded important insights into the nature of the pathways and signals that participate in membrane protein targeting and the overall biogenesis of epithelial polarity.

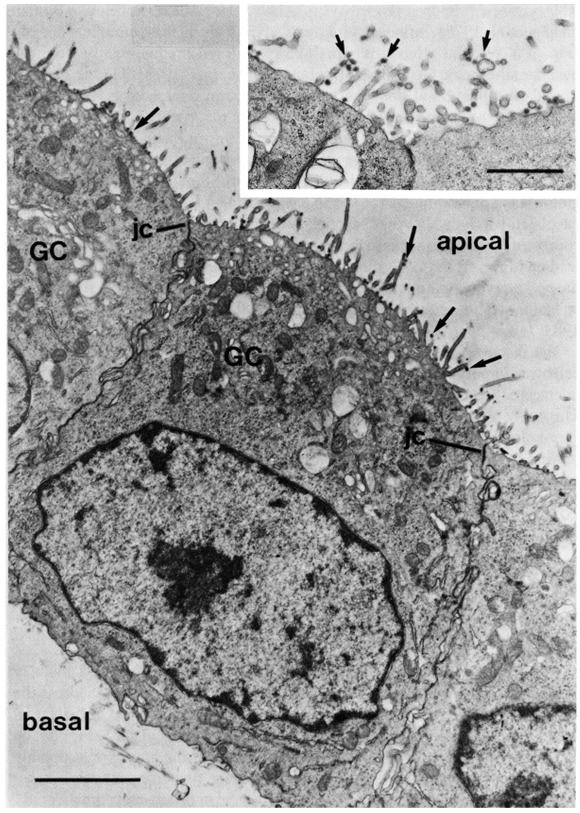

FIGURE 1 The influenza virus buds from the apical surface of MDCK cells. MDCK cells were grown on a hydrated collagen gel, infected with influenza virus for 6 hours, and prepared for electron microscopy. The arrows denote mature virions which assemble at, and bud from, the apical surface. No virus particles are detected at the basal or lateral surfaces. Bar represents 3.0 μm (inset bar represents 1.0 μm). GC, Golgi complex; jc, junctional complex. (Reprinted with permission from Caplan M, Matlin KS. Sorting of membrane and secretory proteins in polarized epithelial cells. In: Matlin KS, Valentich JC, eds. *Functional Epithelial Cells in Culture*. New York: Liss, 1989:71–127.)

Recently, investigators have endeavored to develop new cell lines to study particular aspects of renal cell biology. For example, immortalization genes from human papilloma virus or a hybrid between adenovirus and SV40 have been used to create permanent cell lines from human proximal tubule cells (259, 275). These cell lines are of particular interest because of the proclivity of the proximal tubule to suffer injury following ischemic insult. The cell lines retain differentiated characteristics of the proximal tubule, including expression of brush border markers and sodium dependent/phlorizin-sensitive sugar transport. Cultures of cell lines derived in this fashion are not, however, always able to stably maintain the uniform morphology of a simple epithelium, limiting their usefulness for studies of epithelial polarity.

The study of epithelial cell polarization using cell lines has been facilitated by culturing cells in configurations that more closely resemble in vivo conditions. For example, many varieties of epithelial cells can be grown on permeable filter supports (119, 202). Originally, these were designed to mimic the Ussing chamber used for physiologic studies, but later turned out to also be very useful for biochemical and morphologic experiments as well. In their most common configuration, these supports are composed of polycarbonate filters that form the bottom cup (Fig. 2). The cup is then suspended in a plastic well containing media, and media is added to the inner compartment of the cup. Cells are plated on the upper surface of the filter. When a confluent monolayer is formed, it effectively creates a barrier between the two media compartments. The media in the interior of the cup bathes the epithelial apical surface, whereas the basolateral surface communicates with the exterior media compartment through the pores of the filter. As epithelial cells in the kidney and other organs would normally receive most of their nutrition from the basolateral (serosal) surface, permeable supports are in a sense a more natural growth environment than impermeable tissue culture plastic or glass. Indeed, there is some evidence that epithelial cells are more polarized in filter cultures than on solid substrata (86). Fur-

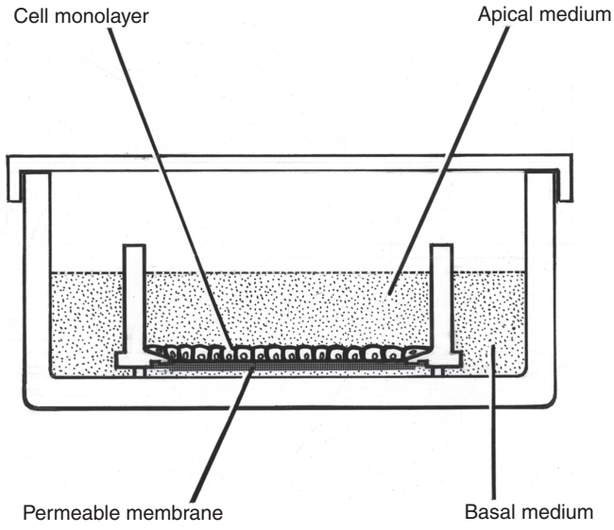

FIGURE 2 Epithelial monolayers can be grown on permeable filter supports. As depicted in the diagram, a porous filter, composed of cellulose acetate or polycarbonate, forms the bottom of a cylindrical cup. Epithelial cells are plated on top of the filter, and the cup is placed in a well filled with media. When the cells become confluent, the resultant monolayer forms a barrier between the media bathing the apical surface and the media in communication with the basolateral surface. This system thus provides investigators with simultaneous and independent access to both plasmalemmal domains.

thermore, the use of filters for the culture of epithelial cells permits investigators simultaneous and independent access to the apical and basolateral plasmalemmal surfaces (187). This useful capacity has been extensively exploited in the experiments described below.

In addition to permeable supports, a number of investigators have now begun to culture renal and other epithelial cell lines embedded in a gel of collagen I or other extracellular matrix molecules. These are called three-dimensional (3D) cultures to distinguish them from more common two-dimensional (2D) cultures on either solid or permeable culture surfaces (Fig. 3) (238, 352) . As with permeable supports, the idea behind 3D cultures is that placing the epithelial cell in an environment in which it is surrounded by an interstitium more closely resembles the in vivo environment. While that conclusion is subject to debate, there is no doubt that certain epithelial phenotypes are more readily expressed in 3D than in 2D cultures (238, 352). Nevertheless, these phenotypes are often slow to develop, frequently taking 7 to 10 days, and may occur asynchronously. This, and the inaccessibility of the cultures somewhat limits their usefulness for biochemical studies. With the advent of high-resolution confocal fluorescent microscopy and the wide array of fluorescent proteins and probes, the impact of this limitation is lessened. Most often, individual suspended cells develop into polarized cysts or, when stimulated with certain growth factors, tubules. As will be described below, use of 3D cultures has led to important fundamental observations about epithelial cell polarization (238, 352).

Adhesion Promotes Epithelial Polarization

Morphogenesis of cells into a polarized epithelium depends on signals from the extracellular environment (70, 225, 269, 282, 344). These signals originate from the attachment of cells to each other and to the substratum, which, in most cases, is the extracellular matrix. If the pattern of cell attachments is asymmetric, then the response of the cell is also asymmetric, and a polar phenotype results. The signals are interpreted by the cell hierarchically (225, 269, 282, 344). Cell adhesion leads to restructuring of the cytoskeleton; junction formation, organization of the cytoplasm and organelles, and sorting of membrane components to the apical and basolateral plasma membrane domains then follow. Although the outline of this complex series of events has been known for some time, until recently only a few of the molecules involved in the process had been identified. As described previously, cell–cell contacts are mediated primarily via E-cadherin and its associated proteins. Cell substratum interactions, on the other hand, are mainly accomplished through integrins interacting with specific extracellular matrix molecules. Following adhesion, signals are generated within the cell that are translated into reorganization of the cytoskeleton, expression of new proteins, and positioning of these proteins into locations within the cell such that their

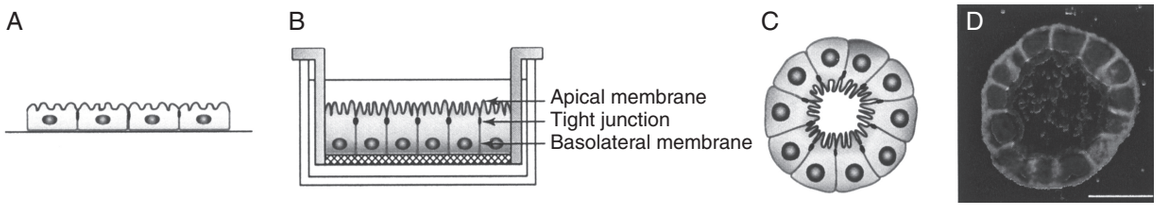

FIGURE 3 Two- and three-dimensional cultures of polarized epithelial cells. Epithelial cell lines may be grown on conventional impermeable substrata such as plastic or glass (A), or on permeable supports (B). In both cases, the provision of a flat, two-dimensional surface may provide spatial signals that normally would be generated by the cells themselves in vivo. In this regard, three-dimensional culture of cells in collagen gels, where a polarized cyst develops over 7 to 10 days (C), may more accurately represent the in vivo environment. In (D), an MDCK cell cyst is fluorescently labeled with antibodies to β-catenin to highlight the basolateral surface. (Reprinted with permission from Zegers MM, O'Brien LE, Yu W, Datta A, Mostov KE. Epithelial polarity and tubulogenesis in vitro. *Trends Cell Biol* 2003;13:169–176.)

function first creates and then maintains the polarized state. Recent research suggests that the mechanisms for generating cell asymmetry are shared by all eukaryotic cells, including simple microorganisms such as yeast and nonepithelial cells such as fibroblasts. What is unique in epithelial cells is not that an axis of polarity is set up within the cell, but that this axis is oriented identically in all interacting cells in the epithelium and is stable as long as the epithelium is not disrupted.

The following sections will summarize current knowledge of the mechanisms of epithelial polarization. As with the polarization hierarchy itself, the presentation will proceed from the proximal adhesive events to signals generated by adhesion and subsequent organization of specific protein complexes believed to be essential for polarization.

Integrins and Other Extracellular Matrix Receptors

The integrins are a superfamily of cell adhesion molecules found in nearly all cells (124, 138, 139). Each integrin consists of a heterodimer of α and β subunits, both of which are transmembrane glycoproteins. A total of 18 α and 8 β subunits are now known in mammals, resulting in at least 24 heterodimers (138). Although integrins are known to be receptors for a variety of extracellular matrix proteins, they may also participate in cell–cell adhesion (124, 138, 139). Epithelial cells of the kidney and other organs typically express an array of integrins including multiple forms with the β1 subunit as well as some with the β3 or β5 subunits (157). The former are most often receptors for collagens and laminins, while the latter are receptors for interstitial or serum proteins such as fibronectin or vitronectin. Many epithelial cells also express integrin α6β4, a laminin receptor (138, 157). The β4 subunit is uniquely found in epithelial cells, and, unlike most other integrins, mediates adhesion through cytokeratins rather than the actin cytoskeleton. The MDCK cell line, for example, expresses α2β1, α3β1, α6β4, and αVβ3 (283). The β1 integrins are receptors for collagens and laminins in these cells, while α6β4 is a receptor for the laminin-5 (LN5) isoform and possibly other laminins (157). Integrin αVβ3 mediates attachment to ligands containing arginine-glycine-aspartate (RGD) sequences such as fibronectin and vitronectin. Recent expression analysis also suggests that integrins containing both the β5 and β6 subunits are also expressed (A. Manninen, University of Oulu, Oulu, Finlar, personal communication, 2006). The complement of integrins expressed varies along the nephron, as does the expression of their extracellular matrix ligands, underlining their involvement not only in cell adhesion but also differentiation (157).

In adherent cells, most integrins mediate adhesion through dynamic interactions with the actin cytoskeleton (94, 348–350). Linkage to actin is mediated by adapter protein complexes that bind to integrin cytoplasmic tails and then to actin. Proteins found in these complexes include talin, which binds directly to integrins and activates their adhesive properties, paxillin, α-actinin, and vinculin (94, 348–350). Studies of migrating cells suggest that initial adhesive interactions occur through small "focal complexes" that form on leading lamellipodia and are linked to polymerizing actin through the action of the small signaling GTPase of the rho family Rac. As the cell moves over these contacts, they mature into larger "focal adhesions" that associate with robust actin stress fibers (at least in culture) controlled by another GTPase Rho and its effectors (94, 348–350). While the general elements of this model have been somewhat validated in epithelial cells during wound healing, the status of focal complexes and focal adhesions in mature polarized epithelia of the kidney and elsewhere, as well as their functions in adhesion and polarization, remain, for the most part, unexplored.

In addition to their role in mechanical adhesion, focal complexes and focal adhesions are also platforms for signaling (94, 348–350). A variety of kinases including, notably, focal adhesion kinase (FAK) and members of the src family of tyrosine kinases, associate with integrin adhesion complexes and are activated by binding to the extracellular matrix. Subsequent signals then activate downstream serine/threonine kinases such as integrin-linked kinase (ILK) and mitogen-activated protein (MAP) kinases such as ERK, as well as members of the rho-GTPase family including RhoA, Rac, and Cdc42. Indeed, at least 50 different cytoskeletal, adapter, and signaling proteins are known to be associated with integrin adhesion complexes, depending on the cell type and circumstances (348, 349).

In addition to the integrins, other membrane proteins are involved in epithelial cell adhesion to the extracellular matrix including dystroglycan, a laminin receptor, and possibly a membrane-bound form of the Lutheran antigen (199, 200). In addition, there is evidence that glycolipids may also serve as transient laminin receptors (169, 353). While not proven that any of these receptors play a direct role in epithelial polarization, they may act indirectly by affecting assembly of the basal lamina (see below) (169).

Cell Adhesion and Development of Primordial Kidney Tubular Epithelium

The developing kidney provides an example of the collaborative roles of cell–cell and cell–substratum interactions in the formation of a polarized epithelium. In the developing kidney, the initial extracellular signal that leads to cell polarization and differentiation of the tubular epithelium arises from mesenchyme following induction by the ureteric bud. The inductive event itself, for which the molecular basis is not understood (232), is isotropic in the sense that is does not impart any spatial information to the differentiation process. The first morphologic indication of differentiation is the formation of multicellular aggregates or condensates. These condensates are spatially differentiated: cells at the peripheries of the condensates have both a "free" plasma membrane domain facing the outside of the condensates and the undifferentiated mesenchyme and an "attached"

plasma membrane domain in contact with other cells of the condensate (Fig. 4). Following this rudimentary polarization, the adherent mesenchymal cells in the condensate become more polarized, eventually reorganizing into a simple epithelium attached to a basal lamina and facing a lumen (Fig. 4). Although cell adhesion molecules are expressed in the undifferentiated mesenchyme, the appearance of the epithelial-specific adhesion molecule E-cadherin coincides with condensation (70, 166, 332). As described earlier, E-cadherin is a member of a family of calcium-dependent cell adhesion molecules found concentrated at the zonula adherens just below the tight junction (312). E-cadherin is present throughout the mesenchyme distributed at the sites of cell–cell contact (70, 332). Despite its coincident expression at the time of condensation, it is unclear whether it plays a key role in the differentiation process. Antibodies against E-cadherin, which disrupt cell–cell contacts in cultured cells, fail to disaggregate or block formation of the epithelium in organ cultures of kidney mesenchyme (332). These results suggest that other cell adhesion molecules of greater significance may await identification.

At a later stage of differentiation, it is likely that the extracellular matrix protein laminin is important in formation of the kidney epithelium from condensate. Laminins are a family of large heterotrimeric glycoproteins found together with collagen type IV, proteoglycans, and other proteins in the basal lamina underlying all epithelia (199, 200, 317). Laminin-1 (LN1), the prototypical molecule of the family, consists of three chains (α1, β1,γ1) associated in a cross-like configuration by disulfide bonding. Although laminin βl chains are expressed in the primitive mesenchyme and in later developmental stages, laminin α1 is first detected following condensation (70, 151, 168, 199). Expression is localized to the periphery of the condensate, suggesting that the crude polarization caused by condensation may have led to polarized secretion of laminin. Antibodies to laminin α1 block formation of the epithelium (151). Thus, assembly of basal lamina containing LN1 around the condensate is essential for differentiation of a polarized epithelium.

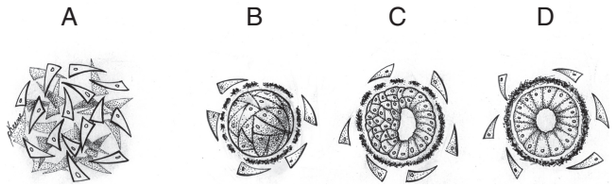

FIGURE 4 Development of the kidney epithelium from induced mesenchyme. The kidney epithelium develops *in vivo* following induction of the metanephric mesenchyme by the ureteric bud. The initial stages of differentiation from mesenchymal to epithelial cells may also be followed *in vitro* by organ culture. In this schematic view, induced mesenchymal cells are initially randomly oriented and show little cell–cell adhesion (A). Some mesenchymal cells adhere closely to each other and begin to produce a basement membrane at the periphery of the condensate (B). The cells of the condensate begin to reorganize into an epithelium and form a lumen as the basement membrane becomes more extensive (C). Finally, formation of the pretubular renal vesicle consisting of a polarized epithelium is complete (D). (Redrawn with permission from Ekblom P. Developmentally regulated conversion of mesenchyme to epithelium. *FASEB J* 1989;3:2141–2150.)

Recent results derived from a variety of other experimental systems generally support the conclusion that laminins and their integrin receptors play a role in epithelial polarization (168). Mutations in either integrin or laminin subunits lead to disruption of epithelial differentiation and polarization in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila* (168). In the mouse embryo, expression of laminin generally coincides with development of epithelial tissues. In embryoid bodies derived from cultures of aggregated embryonic stem (ES) cells, a LN-1-containing basement membrane forms between the endoderm and the polarizing inner cell mass cells. When ES cells deleted of both laminin γ1 alleles are aggregated into embryoid bodies, the inner cell mass forms but does not polarize (168).

The primary receptors for laminins are integrins (157, 197, 199, 200). It is likely that integrins play an important role in morphogenesis and differentiation of the kidney (157). In both the developing and adult kidneys, βl integrins are expressed in a characteristic, cell-type–dependent pattern (147, 154, 157). In uninduced mesenchyme, only the α1 subunit is expressed. As condensates form and epithelialization commences, α6β1, a laminin receptor, appears. Function-blocking antibodies against the α6 subunit inhibit epithelial differentiation in organ cultures of induced mesenchyme, suggesting that this integrin plays a key role. Upon the development of the S-shaped nephron precursor, other α subunits (presumably as heterodimeric complexes with β1) are expressed in a pattern that is retained in the adult (154, 157). Thus, α1 is observed in mesangial cells and the endothelium, α2 is observed in glomerular endothelium and distal tubules, and α3 is observed in podocytes, Bowman's capsule, and distal tubules (154, 157). The α6 subunit is expressed transiently in podocytes during development and basally throughout the tubules from then on (154, 157). Additional evidence for the key roles of integrins in kidney morphogenesis comes from mouse knockouts. Deletion of both α8 (another partner of βl) and α3 integrin subunits affects kidney development (156, 157, 218). Surprisingly, knockout of α6 has no effect on kidney development despite its apparent role in epithelial differentiation in organ culture and its ubiquitous expression in the adult kidney (95). This finding no doubt implies functional redundancy among integrin subunits.

Adhesion and Renal Epithelial Polarization In Vitro

The role of cell adhesion in epithelial polarization is even more clearly indicated through studies with kidney cell lines (269). MDCK cells cultured in a single cell suspension lack polarized plasma membrane domains. Upon attachment to a substratum, cells quickly make cell–cell contacts, forming small islands (230). Under these conditions, apical proteins are restricted to the free or apical surface whereas basolateral proteins are distributed over the entire plasma membrane

(270, 330). As the cells reach confluency, forming a true epithelium, basolateral proteins also become completely polarized (14, 230).

The effects of cell–cell and cell–substratum interactions can be dissected by culturing MDCK cells in medium containing reduced amounts of calcium (231, 330, 331). If cells are cultured on collagen in medium with less than 5 μM calcium, then they attach to the substratum, but formation of cell–cell contacts is inhibited (231, 330, 331). Cells assume a rounded morphology with no appreciable lateral membrane. In this situation, an immature apical surface forms. Microvilli are decreased in number, and expression of apical proteins on the cell surface, although reduced in quantity, remains polarized to the free surface (330). It has also been reported that intracellular concentrations of apical (but not basolateral) proteins, called "vacuolar apical compartments" (VACs), are also present (331). Basolateral proteins, in contrast, are not polarized in medium containing low calcium concentrations. When the calcium concentration is raised to normal values (1.8 mM), then cell–cell contacts rapidly form, VACs exocytose, and basolateral proteins polarize.

The culturing of MDCK cells as multicellular aggregates also permits the effects of cell–cell and cell–substratum interactions to be independently examined (336). Under these conditions, aggregated cells gradually form cysts with central lumina. In the absence of recognizable cell–substratum contact, both apical and basolateral polarization occurs, with the apical surface facing the outside of the cell aggregate. At this time, the tight junctional protein ZO-1 is found distributed over the lateral membrane, where cell–cell contacts occur. As the lumen forms, the cells secrete and deposit type IV collagen and laminin. Interaction with this extracellular matrix then triggers redistribution of ZO-1 to the point of intersection between the apical and lateral membranes (336). The observations with MDCK cells suggest that cell–cell and cell–substratum interactions have somewhat independent, though complementary, effects on cell polarization. In the absence of cell–cell contacts, as in medium with reduced calcium concentrations, cell–substratum interactions are sufficient to induce a degree of apical polarity. Formation of cell–cell contacts then consolidates apical polarity and promotes polarization of basolateral proteins. Conversely, cell–cell contacts in the absence of interaction of cells with a substratum are enough to cause polarization of both apical and basolateral proteins in suspended cell aggregates. The appearance of a collagen substratum, however, affects the localization of tight junctions.

Laminin has also been implicated in the polarization of renal MDCK cells. A monoclonal antibody that blocks MDCK cell adhesion to LN-1, but not collagen, prevents complete polarization (353). Because this particular antibody was directed against a neutral glycolipid Forssman antigen, the significance of this observation was initially uncertain. However, recent results implicating glycolipids in laminin assembly into a basal lamina may help to explain these findings (237, 347). When MDCK cells are cultured in 3D collagen gels for 7–10 days, they form polarized cysts with the apical surface facing the lumen and the basal surface facing the extracellular matrix. Under these conditions, the cells secrete laminin and assemble it into a discrete basal lamina adjacent to the outer surface of the cyst. When MDCK cells are either treated with a function-blocking anti-β1 integrin antibody or express a dominant-negative mutant of the small GTPase Rac1, the laminin-containing basal lamina does not form properly, although the laminin is secreted, and the cells display an inverted and somewhat disorganized polarity (237, 347). Addition of excess exogenous LN-1 to the collagen gel partially rescues both basal lamina assembly and correct polarization, possibly by driving laminin assembly adjacent to the basal plasma membrane (237, 347). The conclusion from these experiments is that the primary defect caused by both the function-blocking anti-β1 antibody and dominant-negative Rac1 is in the laminin assembly, and that an assembled, laminin-containing basal lamina is essential for polarization of MDCK cells in 3D culture.

In summary, the experiments with MDCK cell cysts suggest that a "serpentine" signaling pathway snakes its way from the extracellular matrix in and out of MDCK cells to first signal laminin assembly and then polarization (Fig. 5). The first step in this pathway is interaction of MDCK cells with either the collagen gel or some other matrix molecule through a β1 integrin. Ligation of the integrin then activates Rac1, which then, in a manner that is unclear, leads to laminin assembly on the cell surface. The assembled laminin is then recognized by an integrin or other matrix receptor that in turn signals polarization. As stated earlier, the evidence for this pathway is that both anti-β1 integrin and dominant-negative Rac1 block laminin assembly and polarization, but also that the effects of anti-β1 can be neutralized by overexpression of constitutively active Rac1, and the effects of dominant-negative Rac1 overcome by excess LN-1. While instructive, this pathway is still fragmentary and many questions remain. In MDCK cells, the laminin isoform that makes up the critical basal lamina is likely LN-10 (α5β1γ1), a close cousin of LN-1 (347). However, MDCK cells also synthesize and secrete LN5 (α3β3γ2), a truncated laminin that cannot assemble properly (Mak G, et al., submitted. University of Cincinatti College of Medicine, Cincinatti, Ohio). Whether this molecule plays any role in either basal lamina assembly or polarization in 3D culture is unclear. Furthermore, evidence from 3D cultures of mammary epithelial cells suggests that the epithelial-specific integrin α6β4 may also be involved in polarization (339). However, polarization of MDCK cells, which also express this integrin, are unaffected by function-blocking anti-α6 antibodies (347). Finally, the nature of the polarization signal that apparently depends on the spatial organization of the basal lamina is completely unknown.

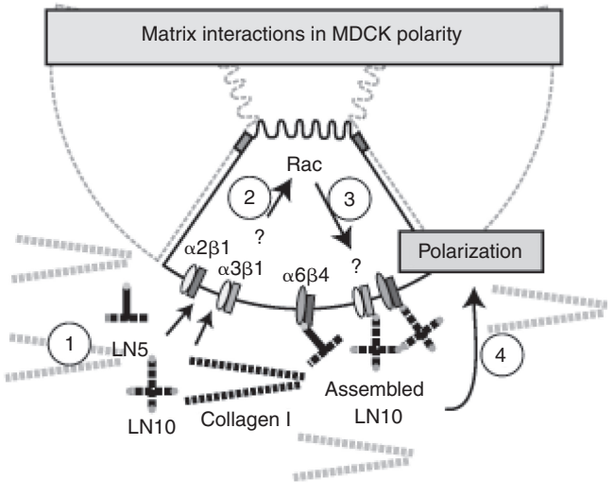

FIGURE 5 Determination of the apical–basal axis in polarizing epithelial cells may depend on a "serpentine" signaling pathway from the extracellular matrix substratum in and out and into the cell. MDCK cells suspended in a three-dimensional collagen gel interact with the collagen, and/or various laminin isoforms via a β1 integrin. This association activates Rac which, in some manner, leads to the assembly of a laminin basal lamina. Signals from this assembled matrix then cause the cells to polarize with the apical plasma membrane in the interior of the cyst and the basal surface on the outside. (From data in O'Brien LE, Jou TS, Pollack AL, Zhang Q, Hansen SH, Yurchenco P, Mostov KE. Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat Cell Biol* 2001;3:831–838; Yu W, Datta A, Leroy P, O'Brien LE, Mak G, Jou TS, Matlin KS, Mostov KE, Zegers MM. Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Mol Biol Cell* 2005;16:433–445; and Mak G, et al., submitted University of Cincinatti College of Medicine, Cincinatti, Ohio.)

Effects of Cell–Cell and Cell–Substratum Interactions on Cytoskeleton

While it is evident that adhesion between epithelial cells and the extracellular matrix substratum elicits both mechanical effects and activates a variety of signaling proteins and intermediates, it is unclear how this information is translated into the formation of the apical–basal axis orthogonal to the adherent surface. Such an axis is essential for establishing the unique identities of the basolateral and apical plasma membrane that define epithelial cell polarity as well as proper assembly and placement of junctional complexes necessary for integrity of the epithelium and its permeability barrier. One possibility is that cortical actin interacts with the microtubule cytoskeleton to affect cell organization. Because of the arrangement of α and β tubulin, microtubules are inherently polar structures. Although microtubule growth may occur from both ends, it occurs faster from the "plus" ends; the "minus" ends associate with microtubule organizing centers (MTOCs) (150). In many cell types, the centrosome is the major MTOC. How the structural polarity of microtubules might be used to translate signals at the cell periphery into asymmetric morphogenesis is suggested by the dynamic instability model of Kirschner and Mitchison (Fig. 6) (150). According to this model, microtubules growing from initiation sites (MTOCs) spontaneously and suddenly disassemble. As the microtubule extend and contract, each transient configuration represents a potential cell morphology that is only realized if factors are present that stabilize particular microtubule configurations and prevent disassembly. Candidates for such stabilization factors are MAPs, including capping proteins that might bind the "plus" ends and MAPs that associate with the sides of the microtubules (150). If such factors bind to a particular locale within the cell cortex, then microtubules extending to this region will be stabilized and preserved (Fig. 6). A line extending along the stable microtubules from the point of capping in the cortex to the point of initiation at the MTOC would represent a primitive axis around which cell polarity might develop (Fig. 6).

Microtubule dynamics in MDCK cells are certainly consistent with this model. The microtubule cytoskeleton in MDCK cells undergoes dramatic rearrangement and stabilization as cultures progress from subconfluency to confluency and cells become more polarized (Fig. 7) (13, 28). In subconfluent cultures, the microtubules originate primarily from the juxtanuclear centrosome (Fig. 7). As the cells form contacts and become more confluent, the centrosome moves first to the periphery of the cell (where it no longer organizes microtubules) and ultimately resides in the center of apical surface (where it may act as a basal body for central cilium) (Fig. 7). At the same time, the microtubule cytoskeleton becomes polarized along the emerging apical-to-basal axis (Fig. 7) (13, 28). When the cells are fully polarized, microtubules are organized into a dense apical cap and also run along the length of the cell, with their minus ends apical and their plus ends basal (Fig. 7) (13). A network of tubules is also found on the basal surface (13, 262). Several lines of evidence also suggest that the microtubules found in fully developed monolayers are also greatly stabilized (28, 29). Furthermore, observations in fibroblast cell lines suggest

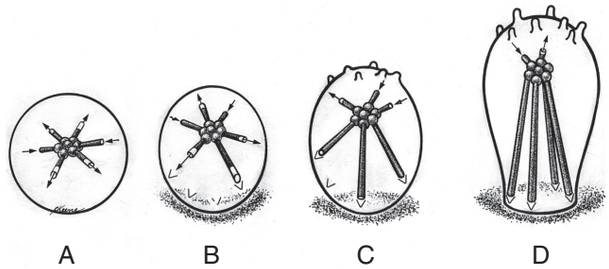

FIGURE 6 Morphogenesis of a polarized epithelial cell through stabilization of particular microtubules. Microtubules nucleated by a central microtubule organizing center (MTOC) grow and contract spontaneously, as if testing different configurations (A). Association of the cell with basal lamina (B) leads to the binding of factors (*V*) to the cell cortex in the regon where adhesion occurs. When microtubules growing toward the region of substratum contact encounter the factors, they are stabilized and an axis perpendicular to the substratum is created, which leads to morphogenesis of polarized cell (D). The polarity of microtubules seen in MDCK cells is consistent with the model (see Fig. 7). (Redrawn with permission from Kirschner M, Mitchison T. Beyond self-assembly: from microtubules to morphogenesis. *Cell* 1986;45:329–342.)

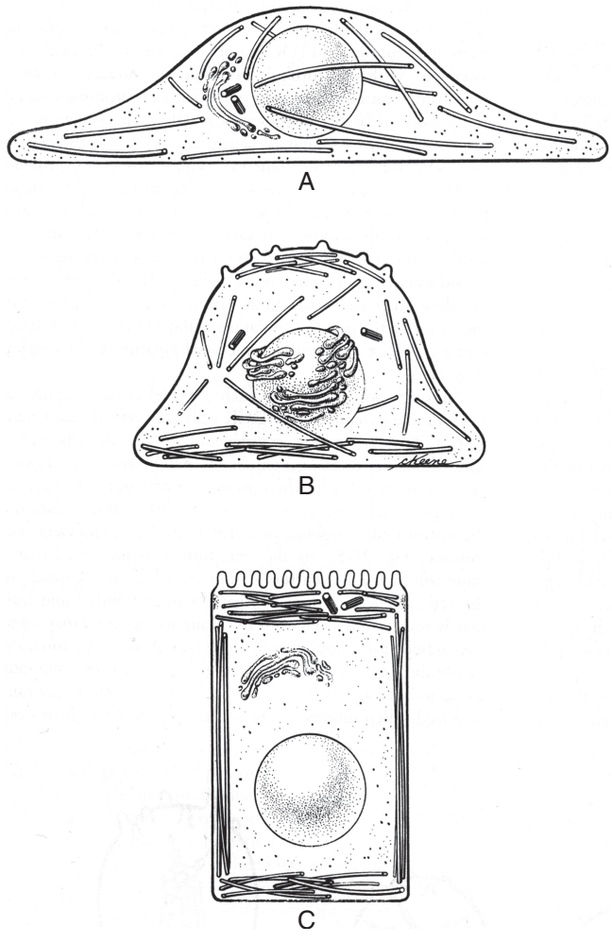

FIGURE 7 Reorganization of microtubules in MDCK cells. In sparsely confluent cultures of MDCK cells, microtubules are nucleated by a juxtanuclear centrosome lying near the Golgi complex (A). As the cultures mature the microtubule organization is no longer nucleated from the centrosome. The centrioles split and move toward the apical lateral borders of the cell. At the same time, the microtubules begin to form an apical cap (B). When fully confluent and polarized, the centrioles are positioned under the apical plasma membrane, where they may serve as a basal body for central cilium. The microtubules are highly organized with a dense apical cap and vertical microtubules running along the lateral borders. Some microtubules are also found on the basal surface (C). The orientation of the vertical microtubules is with the minus-end apical and the plus-end basal, suggesting that organizing centers are found in the apical region. Note also that movement of the Golgi complex from the juxtanulear location to the apical cytoplasm (compare B and C). (From results Bacallao R, Antony C, Dotti C, Karsenti E, Stelzer EHK, Simons K. The subcellular organization of Madin–Darby canine kidney cells during formation of a polarized epithelium. *J Cell Biol* 1989;109:2817–2832; and Bre MH, Kreis TE, Karsenti E. Control of microtubule nucleation and stability in Madin–Darby canine kidney cells: the occurrence of noncentrosomal, stable detyrosinated microtubules. *J Cell Biol* 1987;105:1283–1296.)

that focal adhesions may be a site where growing microtubules are captured and stabilized (145).

Work over the past few years has identified a number of proteins involved in the capping and organization of microtubules in polarized epithelial cells. Chief among these is APC, the protein also known as a regulator of Wnt signaling and β-catenin degradation described previously (203, 262). Careful studies by Näthke and others in highly polarized epithelial cells from the inner ear, which possess bundles of microtubules oriented along the apical–basal axis, clearly established that APC is found exclusively localized to the basal surface under these conditions, near the plus ends of microtubules (203). APC interacts with microtubules directly through a C-terminal–binding site, but also probably interacts indirectly at its N-terminus through armadillo repeats that bind a kinesin-associated protein (KAP3) (203, 262). The N-terminus of APC also binds Asef, a Rac-GEF, that may facilitate APC association with the basal actin cortex (203, 262). This could provide a potential linkage between basal adhesion complexes and microtubules. Other plus-end microtubule-binding proteins, such as EB-1 and $p150^{Glued}$, associate also with the sides of microtubules, as does APC, and influence microtubule dynamics in the basal cortex (262). All of these proteins may also help link microtubules via actin to the lateral plasma membrane as epithelial cells polarize, facilitating development of the fully polarized cell. Despite these intriguing and relevant observations, involvement of dynamic microtubules in the establishment of the apical–basal axis is far from proven. In cultured epithelial cells such as MDCK and Caco-2, the relationship between an oriented microtubule cytoskeleton and localization of key microtubule-binding proteins such as APC is far from strict (110, 203). It is likely that microtubule dynamics represent but one of many redundant mechanisms responsible for establishing the epithelial apical–basal axis in what must, by necessity, be a very robust process.

While the microtubule cytoskeleton may help to establish the apical–basal axis, cell–cell adhesion clearly affects organization of the cortical cytoskeleton on the lateral plasma membrane. How this may occur may be inferred from studies of the submembranous cytoskeleton composed of fodrin, ankyrin, and actin (226). Fodrin is an analogue of erythroid spectrin found in many nonerythroid cell types (22). In polarized kidney cells, fodrin is associated exclusively with the cytoplasmic side of the basolateral surface (65, 230). In MDCK cells, fodrin forms a complex with ankyrin and Na^+,K^+-ATPase (211, 227). This suggests that the organization of the fodrin-based cytoskeleton in epithelial cells might resemble that seen in the erythrocyte, where ankyrin acts to link the network of spectrin and actin to the membrane by associating with both spectrin and the anion transport–protein band 3 (22, 66). In polarized MDCK cells, fodrin is found in a metabolically stable and biochemically insoluble state (62, 230). In contrast, fodrin is diffusely distributed in the cytoplasm of newly plated MDCK cells, and in cells cultured in medium containing reduced calcium concentrations, basolateral polarity is lacking (62, 230, 231). Under these conditions, fodrin exists in metabolically unstable complexes that are extractable with nonionic detergent (62, 230, 231). In addition to fodrin, ankyrin, and Na^+,K^+-ATPase, these complexes also con-

tain E-cadherin (228). Because of this, it has been postulated that upon cell–cell contact, E-cadherin induces assembly of the fodrin cytoskeleton (227, 228, 269). In this manner, the fodrin cytoskeleton would form only on regions of the membrane where cell–cell contacts occur, namely, the lateral plasma membrane domain. Polarization of membrane proteins might then come about either by ankyrin-mediated redistribution or by endocytosis and degradation of proteins misplaced to the incorrect domain. Elements of this hypothesis are supported by the observation that expression of E-cadherin in nonpolar cells causes endogenous fodrin and Na^+,K^+-ATPase to be localized exclusively to the membranes involved in E-cadherin expression, suggesting that more than one molecular mechanism may be important in the development of the basolateral domain (196).

Par Proteins and Establishment of Apical and Basolateral Membrane Identity

Although the importance of cell adhesion and epithelial polarization is clear, the detailed mechanisms by which this occurs are undoubtedly very complex. Studies of early development in the model organisms *C. elegans* and *Drosophila* identified a number of genes responsible for the asymmetric partitioning of cell fate determinants during cell division and establishment of the anterior–posterior axis (176, 225). In some cases, these genes controlled the orientation of the axis of cell division, which is clearly related to epithelial polarity where cell division may either yield two identical epithelial daughter cells or one epithelial cell and a new cell type. Mutants in such genes were termed "PAR" for partition defective. Of the six genes originally identified in *C. elegans*, five along with the small GTPase Cdc42 and an atypical isoform of protein kinase-C (aPKC) have been implicated in polarization of mammalian epithelial cells (176, 225). These include PAR1 (in mammals also called MARK/CTAK/KP78/EMK), PAR3 (Bazooka in *Drosophila*), PAR4 (in mammals LKB1/STK11), PAR5 (in mammals an isoform of the phosphoserine-binding protein 14-3-3), and PAR6.

Based on work primarily in *Drosophila*, some PAR proteins appear to associate with other proteins into three complexes that function together to establish apical and basolateral membrane domains and properly position junctional complexes, while others function independently (26). These are PAR3, PAR6, and aPKC (Baz or Par complex), Crumbs, PATJ, and Stardust (PALS) (Crb complex), and lethal giant larva (LGL), Scribble, and discs large (DLG) (Scrib complex). Each of these complexes has at least one component with a PDZ domain that presumably facilitates their association with the junctional region. In *Drosophila*, the Baz complex initiates apical polarity after being recruited to the adherens junction. The Scrib complex binds to the basolateral membrane and represses apical identity in this region, while the Crb complex, recruited apically by the Baz complex, antagonizes Scrib and reinforces the "apicalization" effects of Baz (26).

How these protein complexes may function in mammalian epithelial cells is currently under intensive investigation (176). The small GTPase Cdc42 is of particular interest because it has long been implicated in polarized functions such as bud site selection in yeast and in establishment of transient polarity during neutrophil chemotaxis (176). Furthermore, Cdc42 has also been implicated with PAR6 in directional migration of astrocysts and wounded endothelial cell monolayers (176). How it might function in the context of epithelial polarization is not clear. Evidence from both *Drosophila* and MDCK cells suggests that aPKC, PAR1 (also serine/threonine kinase), and PAR5 (14-3-3) might function together to restrict certain protein complex functions to the apical membrane (26, 176). In particular, phosphorylation of PAR1 by aPKC generates a binding site for 14-3-3 that restricts its location to the basolateral surface (23, 136, 137, 308, 309). PAR1 may then phosphorylate PAR3 to also create a 14-3-3 binding site that prevents its association with PAR6, thereby limiting its the activity of the PAR3/PAR6 complex to the nascent apical surface.

Despite these clues, other observations hint at the complexity of the polarization process and possible differences between mammals and invertebrates. PAR3 is essential for epithelial polarization in *Drosophila*, for example (26, 176). Knockdown of PAR3 in MDCK cells using RNA interference, however, significantly affects tight junction formation but not necessarily polarity (47, 48). Furthermore, this function of PAR3 in MDCK cells is apparently independent of its interaction with PAR6 or aPKC. Suppression of PAR3, at the same time, constitutively activates Rac, and a Rac-dominant negative mutant rescues tight junction formation (47, 48). Similarly, PAR4, a serine/threonine kinase, was implicated in polarization in *C. elegans* and *Drosophila*. When the mammalian PAR4 homologue LKB1 is activated in an intestinal cell line by inducible-expression of the adapter STRAD, individual cells form an apical surface and localize junctional proteins adjacent to this surface in the absence of any cell–cell contacts, despite the fact that LKB1 itself is not distributed in a polarized fashion (12). Thus, some as yet undefined markers of asymmetry remain to be identified that are critical in initiating the cascade of events leading to polarization.

SORTING PATHWAYS

One of the first, and perhaps most easily addressed questions presented by the phenomenon of epithelial polarity relates to where, within the cell, sorting occurs. The membrane proteins that populate the apical and basolateral plasmalemmal domains are all synthesized in association with the membranous elements of the rough ER (335). It has further been shown that after their cotranslational insertion into the membranes of the rough ER, apically and basolaterally

directed proteins share the same cisternae of the Golgi complex as they transit the secretory pathway en route to their respective sites of ultimate functional residence (85, 263). Immunoelectron microscopic studies performed on MDCK cells doubly infected with the VSV and influenza viruses revealed, through double labeling, that the influenza HA protein and the VSV G protein could be colocalized throughout the cisternae of the Golgi complex (263).

This observation was confirmed and extended through a series of elegant biochemical studies. It had previously been shown that when cells are incubated at 20°C newly synthesized membrane proteins accumulate in the trans-most cisterna of the Golgi complex (186, 278). Elevating the temperature to 37°C relieves this block and allows the proteins to proceed to the cell surface (108). By examining the nature of the complex N-linked glycosylation associated with the VSV G protein, it was demonstrated that sialic acid residues are added in the 20°C compartment (85). These investigators took advantage of the fact that, in addition to the HA protein, the membrane of the influenza virus contains a neuraminidase in their efforts to determine whether segregation of the apically directed influenza proteins from the basolaterally targeted VSV G protein occurs before or after the 20°C block compartment. They found that in singly infected cells incubated at 20°C, the VSV G protein became heavily sialylated. In contrast, when cells that had been doubly infected with both the VSV and influenza viruses were incubated at 20°C, little if any sialic acid could be detected on the newly synthesized VSV G protein. These results demonstrate that as late as the 20°C block compartment, which corresponds to the trans-most cisterna of the Golgi complex, the newly synthesized apical neuraminidase and basolateral VSV G protein are still intermingled and capable of physical interaction. The segregation of these two classes of proteins from one another must, therefore, occur at or after this subcellular locus. It is interesting to note that immunoelectron microscopic studies of endocrine cells reveal that proteins destined for packaging in secretory granules are separated from those bound for constitutive delivery to the cell surface in the trans-most cisterna of the Golgi complex (246, 319, 320). Observations such as these have prompted investigators to speculate that this compartment, which is also referred to as the trans-Golgi network (TGN), might be the site of several intracellular sorting events (109). More recently it has been shown that sorting may occur as well at the level of the recycling endosome. Loading endosomes with HRP (horseradish peroxidase)-conjugated transferrin and subsequently disrupting endosome function through the deposition of peroxidase reaction product prevents the surface delivery of newly synthesized basolateral membrane proteins (7).

Three pathways for this sorting process can be imagined (Fig. 8) (39, 184, 269, 293). In the direct model, sorting would take place prior to cell surface delivery. Segregation of basolateral from apical proteins would be completed intracellularly and proteins would never appear, even transiently, in the inappropriate membrane domain. The random sorting scheme dictates that no separation of apical from basolateral proteins occurs prior to arrival at the cell surface. Following their insertion into the plasmalemma, proteins that find themselves in the wrong surface domain would be removed by endocytosis and either transcytosed to the proper surface (185) or degraded. Finally, the indirect paradigm predicts that all newly synthesized plasmalemmal proteins initially appear together either at either the apical or basolateral membrane. The proteins for which this delivery is correct would be retained in that membrane domain, while those that had been mis-delivered would be internalized and transcytosed to their sites of ultimate functional residence.

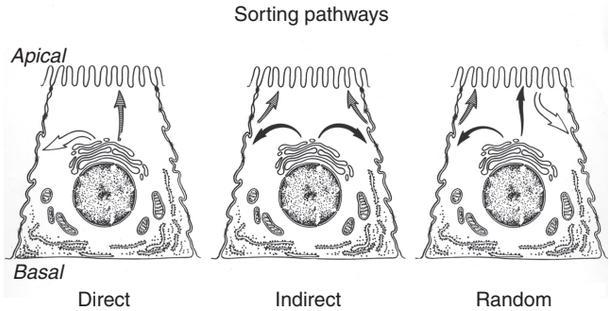

FIGURE 8 Three putative pathways for the sorting of membrane proteins in polarized epithelial cells. In the vectorial sorting scheme, apical and basolateral membrane proteins are separated from one another intracellularly and prior to plasmalemmal delivery (left). The indirect, or obligate, misdelivery model predicts that all newly synthesized plasma membrane proteins are carried together to cone–cell surface domain. Proteins destined for the opposite surface are then internalized and transported to their appropriate destinations (middle). Finally, random sorting is defined by a complete lack of intracellular segregation. Apical and basolateral proteins are delivered without preference to both surfaces and are subsequently redistributed by endocytosis and transcellular transport (right). Clear arrows represent vesicles carrying only basolateral proteins, hatched arrows denote vesicles carrying only apical proteins, and black arrows indicate vesicles carrying intermixed apical and basolateral membrane proteins. (Reprinted with permission from Caplan M, Matlin KS. Sorting of membrane and secretory proteins in polarized epithelial cells. In: Matlin KS, Valentich JC, eds. *Functional Epithelial Cells in Culture*. New York: Liss, 1989:71–127.)

These three models, although perhaps somewhat simplistic, are valuable for the relative ease with which they can be experimentally distinguished. Over the past two decades a great deal of effort has been invested in identifying which of these routes is, in fact, operational. The rather surprising answer appears to indicate that the sorting pathway pursued varies among different cell types and even among different proteins within the same cell type.

Technical Approaches

Much of the early research into the nature of epithelial sorting pathways was carried out on MDCK cells that have been infected with the VSV or influenza viruses. The infected cells produce massive quantities of viral proteins and

retain their polarized distribution throughout at least the initial stages of the infection. These properties greatly facilitate the detection of cohorts of newly synthesized membrane proteins in the pulse chase protocols generally employed to monitor the polarity of cell surface delivery. Pulse labeling experiments demonstrated that the VSV G protein is not accessible to apically added antibodies at any point during its postsynthetic processing (253). In the case of the influenza HA protein, the converse is true. Proteases (187) or antibody probes (201) added to the media compartment bathing the basolateral surfaces of MDCK cells grown on filters cannot cleave or interact with this polypeptide during its journey to the apical cell surface. From these results it was concluded that the direct model of sorting applies for at least these two proteins in MDCK cells.

Other labeling tools have also been brought to bear on the study of sorting pathways. The N-hydroxysuccinimidyl (NHS) derivative of biotin is a membrane-impermeable molecule that will covalently combine with the ϵ-amino groups of exposed lysine residues (279). Proteins thus modified are substrates for precipitation or detection with avidin-conjugated secondary reagents. These tools can be used to follow the fate of large numbers of membrane proteins that have been exposed at one or the other cell surface to the NHS biotin compound. Using such a protocol, it has been demonstrated that several MDCK-cell apical and basolateral membrane proteins are directly targeted to their appropriate membrane domains (162, 174). Similar results have been gathered for an adenocarcinoma cell line (161).

Sodium Pump Targeting

Further support for the vectorial paradigm in MDCK cells came from studies on the sorting of the endogenous Na^+,K^+-ATPase (40, 41). Filter-grown MDCK cells that had been pulse-labeled with [35S]-methionine were exposed to the N-azidobenzoyl (NAB) derivative of ouabain at either their apical or basolateral surfaces during the course of a 90-minute chase. NAB-ouabain will bind to catalytically active sodium pumps with high affinity and, following UV photolysis, will become covalently incorporated into the protein backbone of the Na^+,K^+-ATPase's α subunit (42, 79, 80). By analyzing immunoprecipitates prepared from these cells using an anti-ouabain antibody, it was possible to demonstrate that no sodium pump in a state competent to bind ouabain ever appears at the apical surface.

Another investigation of sodium pump sorting in a different clonal line of MDCK cells made use of the NHS biotin surface-labeling technique and arrived at a conclusion diametrically opposed to the one described above. The results of this study indicated that the Na^+,K^+-ATPase is randomly delivered to the apical and basolateral plasmalemmal surfaces (116). The authors further suggested that stabilizing interactions with cytoskeletal elements that underlie the basolateral but not the apical cell surface (211, 227, 231) result in a much longer residence time for pump inserted into the basolateral domain. These studies are thus consistent with a model in which the sodium pump is not sorted intracellularly, but instead achieves its basolateral distribution through a mechanism based on random delivery followed by differential stabilization.

The experiments of Hammerton et al. (116) made use of NHS biotin as the membrane-impermeable covalent tag (279) with which to monitor the cell surface delivery of the newly synthesized sodium pump. Using this approach, these investigators found that newly synthesized Na^+,K^+-ATPase labeled during a 1-hour pulse was available to biotinylation from both the apical and basolateral surfaces in roughly equal proportions. This experiment was subsequently repeated employing a similar protocol with minor modifications and using the same clone of MDCK cells as had been used in the NAB-ouabain study. Newly synthesized Na^+,K^+-ATPase could be detected at the basolateral surface as early as the 30-minute chase point. Less than 5% of the total radiolabel led sodium pump was biotinylatable from the apical surface at any of the chase intervals employed in this study (102). E-cadherin, another basolateral membrane protein (228), was also found to appear exclusively at the basolateral surface. In contrast, a 114-kDa apical protein (14) could only be biotinylated from the apical surface at each time point, demonstrating that the NHS-biotin reagent does, in fact, have access to this cell surface domain. Thus, these results demonstrate that newly synthesized Na^+,K^+-ATPase is sorted intracellularly and targeted directly to the basolateral surface. This observation is consistent with the previous studies employing NAB-ouabain (41) and was corroborated by the similar studies performed on thyroid epithelial cells (356). Finally, it is important to note that subsequent studies have made use of the NHS-biotin technique to compare the delivery of Na^+,K^+-ATPase to the cell surface in the two different MDCK cell clones alluded to above (195). This study found that the cell line associated with random delivery once again produced this result, whereas the cell line in which vectorial delivery had been detected once again exhibited vectorial delivery. Thus, the apparent discrepancy among these studies appears to be attributable to differences in the pathways and processes through which these closely related cell lines achieve the polarized distribution of the Na^+,K^+-ATPase. While one line targets the pump directly to its basolateral destination, the other delivers it randomly and depends on cytoskeletal interactions to stabilize only the basolateral pool. Clearly, therefore, while cytoskeletal interactions may be sufficient to localize the Na^+,K^+-ATPase to the basolateral surface, they are clearly not the sole mechanism involved in producing the sodium pump's anisotropic distribution. Instead, they may act as a failsafe mechanism to back up and reinforce the initial biosynthetic sorting of the Na^+,K^+-ATPase to ensure that its polarized distribution is attained and maintained.

The preceding discussion suggests that the direct scheme cannot be applied to all epithelia or even to all MDCK cell clones. An alternate system has been shown to apply to the

liver, for example. Cell fractionation studies performed on liver by Bartles et al. (18) reveal that several apical membrane proteins appear in the fraction corresponding to the hepatocyte basolateral plasma membrane prior to being delivered to the apical surface. This route has been especially well documented for the polymeric immunoglobulin receptor (pIgR) expressed by hepatocytes. This 120-kDa polypeptide serves to carry dimeric IgA from the blood to the lumena of the bile canaliculi. During its biosynthesis, the pIgR is transported directly from the TGN to the basolateral cell surface where it is available to bind dimeric IgA (129, 311). Independent of any interaction with IgA, the receptor becomes phosphorylated in the basolateral plasmalemma, and the phosphorylated form is internalized and carried by a transcytotic vesicle to the apical, or canalicular, surface (159). Following its insertion into the apical plasma membrane the ectodomain of the pIgR is cleaved and released into the bile as an 80-kDa protein referred to as secretory component (129, 311). Association with the secretory component helps to protect the bound IgA from intestinal proteases. Coupled with other results (14), the behavior of pIgR in hepatocytes supports the contention that apical membrane proteins arrive at their site of ultimate functional residence via obligate mis-delivery to the basolateral domain. This paradigm may not apply to all apical proteins in hepatocytes. Studies of the trafficking of apical members of the multidrug-resistance family of transport proteins indicate that these polytopic membrane proteins do not make an appearance at the basolateral surface en route to the apical membrane (149, 334). Thus, within a single polarized cell type, multiple trafficking routes can be employed to target different proteins to the same place.

A combination of the direct and indirect paradigms seems to be involved in membrane protein delivery in cultured intestinal cells. The Caco-2 line of human colon carcinoma cells can be grown on filters and subjected to the NHS-biotin labeling protocol described above. Such experiments reveal that the basolateral protein followed is vectorially targeted (190). Analysis of the apical polypeptides produced a somewhat more complicated picture. A fraction of these proteins appeared to transit through the basolateral plasmalemma prior to their apical delivery. The remainder of the apical proteins studied in this sampling were sorted intracellularly and inserted directly at the apical domain. Related and somewhat more complicated results have been gathered from studies on the biogenesis of brush border hydrolases by colonocytes in situ (2, 121, 182).

To complete this already confusing picture it is necessary to return to a discussion of targeting studies in MDCK cells. A cDNA encoding the pIgR has been expressed by transfection in this cell line. Remarkably, the sorting pathway pursued by this protein in the cultured renal epithelium is apparently identical to the rather baroque scheme that characterizes its route in hepatocytes (214). From the TGN the pIgR travels to the basolateral surface, from which it is internalized and subsequently transcytosed to the apical pole or recycled to the basolateral side. These observations demonstrate that an obligate mis-delivery pathway is either created or simply revealed in MDCK cells expressing the pIgR.

This apparent diversity of sorting pathways is perhaps not as surprising as it first appears. The relative flow of membranous vesicles from the Golgi complex to the two plasmalemmal surfaces in different epithelial cell types is likely to reflect a cell's biologic mission as well as the nature of the environment in which it functions. It appears, for example, that although hepatocytes produce copious quantities of secretory proteins, none are released directly into the bile (132). It has been proposed that newly synthesized membrane proteins depart the Golgi in the same transport vesicles that carry proteins destined for constitutive secretion (132, 293). Were this the case, then cells that do not produce a secretory content targeted for one or another membrane domain may also lack direct traffic of membrane vesicles directed from the Golgi to that domain. The full complement of plasmalemmal proteins might thus be forced by default to share the same carrier out of the Golgi and to be sorted by transcytosis subsequent to cell surface delivery. Some hepatic apical membrane proteins may transit through the basolateral surface because there is very little nonstop cargo traveling from the TGN to the apical domain in this particular cell type. The apparent multiplicity of sorting pathways available to different proteins within the same cell type may reflect specializations relevant to these proteins' functions. Diversity may also arise from nature of the signals and mechanisms that mediate these proteins' polarized distribution. The potential contribution of this latter influence will be referred to again in sections to follow. The lack of a single answer or unifying solution to the problem of sorting pathways is a theme that carries through the entire study of epithelial polarity. A number of equally effective mechanisms appear to have evolved for segregating membrane proteins into distinct domains. It remains to be determined how these differing approaches benefit their respective tissues and contribute to the maintenance of their unique functions.

Sorting Signals

Rodriguez-Boulan and Sabatini's 1978 observation that viral spike glycoproteins are targeted to opposite domains of polarized epithelial cells (267, 268) gave rise to the hypothesis that sorting signals—that is, the information required to direct a protein or proteins to a given subcellular location—might be wholly contained within the structure of the sorted proteins themselves. Evidence in favor of this contention has come from studies examining the distribution of viral membrane proteins expressed by transfection (rather than infection) in polarized cultured cells. A number of investigators have shown that the influenza HA protein, the VSV G protein and related viral spike glycoproteins are sorted correctly in the absence of any other proteins encoded by viral genomes (103, 144, 271, 302). It is apparent, therefore, that all

of the addressing information necessary to produce the polarized distributions of these polypeptides must be embodied within the proteins themselves. It has further been shown that this information is almost certainly associated with the protein backbone rather than with any post-translational modification. Cells whose capacity to add asparagine-linked sugar residues has been impaired, either through mutation or via treatment with tunicamycin (106, 272), are nonetheless able to correctly target the viral spike proteins. Observations such as these have sparked an intensive search for the actual molecular information that specifies localization and for the machinery that acts on this information. It must be stated at the outset, however, that despite the rather confident and declarative tone of this section's heading, the identification and characterization of epithelial-sorting signals and mechanisms is still in its infancy.

Several distinct classes of signals have been found to specify basolateral sorting. Perhaps the best characterized of these are short motifs that contain tyrosine residues and resemble or overlap with sequences involved in endocytosis. Work from a number of groups has suggested that sequences in the cytosolic tail of membrane proteins determine the rates at which these proteins are internalized. The presence of a tyrosine residue appears to be a critical determinant of the efficacy of an endocytosis signal (62). The rapid endocytosis of both the LDL receptor and the transferrin receptor, for example, is dependent on the presence of short, tyrosine-containing sequences in these proteins' cytoplasmic tails. Mutation of this tyrosine residue to any other amino acid vastly reduces the rates at which both of these proteins are internalized. The apically sorted influenza HA protein is normally endocytosed extremely slowly. Addition of a tyrosine residue to the cytosolic tail of the influenza HA protein causes it to behave like the LDL receptor or transferrin receptor with respect to endocytosis—that is, it is rapidly internalized and recycled (160). When this altered form of the HA protein is expressed in MDCK cells, it is detected predominantly at the basolateral plasma membrane (35). It would appear, therefore, that a signal which is permissive for endocytosis is also competent to mediate basolateral accumulation.

Studies of the VSV G protein reveal that its basolateral sorting is also driven by a tyrosine-containing motif (314, 315). Uptake measurements suggest, however, that the VSV G protein is internalized relatively slowly, suggesting that its tyrosine-based motif confers basolateral targeting but not rapid endocytosis. Mutagenesis studies of the tyrosine-modified influenza HA protein as well as several other basolateral membrane proteins indicate that while internalization signals and basolateral sorting signals can share the same critical tyrosine residues, they are not identical (171). Altering residues near the tyrosine can produce apically sorted influenza HA protein that is rapidly endocytosed and basolateral HA protein that is internalized only slowly. Thus, basolateral and endocytosis signals can overlap, sharing one or more residues, but are clearly distinguishable from one another. Presumably, therefore, they must be interpreted by distinct cellular machinery.

Data pointing to a similar conclusion have been gathered for Fc receptors (135). One of the Fc receptor isoforms includes a di-leucine sequence in its cytoplasmic tail. This sequence has been shown to function as an endocytosis signal and it also appears to confer basolateral targeting when the protein is expressed in polarized cells. Once again, alteration of residues flanking the di-leucine motif demonstrates that the sequence requirements for basolateral sorting are distinct from those that specify internalization (133, 192).

Tyrosine-containing basolateral sorting signals that are entirely distinct from recognizable endocytosis motifs have also been detected. The LDL receptor depends on a basolateral sorting signal that bears no sequence resemblance to any known internalization motif (134, 192). Although this motif includes a tyrosine residue, mutation of that tyrosine to phenylalanine still permits basolateral localization. A distinct tyrosine-containing motif appears to mediate the internalization of the LDL receptor (191). In the absence of the primary basolateral signal, this endocytosis motif can mediate a basolateral sorting function. Once again, however, with the exception of the tyrosine residue, the amino acids that contribute to the basolateral and endocytic aspects of this signal are distinct from one another.

Several basolateral sorting signals unrelated to tyrosine residues have also been reported. The well-characterized tyrosine-based endocytosis motif of the transferrin receptor is completely distinct from this protein's basolateral targeting signal, which resides in a different portion of the cytoplasmic tail. The peptide-processing enzyme furin cycles between the trans-Golgi network and the basolateral plasmalemma (239). Its trafficking to the basolateral surface appears to be driven by residues that are associated with a casein kinase-II phosphorylation site (143, 289). The invariant chain of the major histocompatibility class I1 complex is sorted to the basolateral membrane by virtue of the dihydrophobic sequence Met-Leu (240). Once again, endocytic internalization of this molecule is conferred by a similar dihydrophobic sequence, Leu-Ile, which is present at another position on the cytoplasmic tail. All of the basolateral sorting motifs discussed thus far function in the context of membrane proteins that span the bilayer once. As will be discussed below, a completely different cadre of molecular sequences appears to mediate the targeting of ion transporters and other multispanning membrane proteins. The list of identified basolateral sorting signals is considerably more extensive than the inventory of characterized apical-membrane protein-sorting signals. Perhaps the best studied member of this latter roster is not, in fact, a protein-based signal at all, but is instead constituted entirely of phospholipid. Glycophospholipid (GPI)-linked proteins are synthesized as transmembrane polypeptides that are cotranslationally inserted into the membrane of the rough ER (61). While still associated

with the ER, the GPI-linked protein's ectodomain is proteolytically removed and transferred to a preassembled structure composed of a complex glycan tethered to the membrane through its attachment to a molecule of phospholipid (frequently phosphotidylinositol). Previous work has shown that in polarized epithelial cells, essentially all of the GPI-linked proteins reside in the apical plasmalemma (173, 174). Interestingly, the apical surface also plays host to the cell's full complement of glycolipid (327). Investigators prepared a construct in which the VSV G ectodomain was wedded to the transmembrane tail of Thy-1, which carries a signal for glycophospholipidation (37). The resultant GPI-linked G protein is sorted to the apical membrane. Similar results have been gathered by another group using a different construct. The results of these and related experiments have generally been interpreted to indicate that a strong apical sorting signal is embodied in some component of the GPI linkage itself. The transmembrane domains of several single-spanning apical membrane proteins appear to carry information important for apical targeting. The transmembrane domains of the influenza virus neuraminidase and HA proteins, for example, are sufficient to mediate sorting to the apical surface when they are included in constructs expressed by transfection in MDCK cells (158, 220). As will be discussed below, the same mechanisms that are thought to be involved in recognizing the GPI tail as an apical sorting motif may also interpret signals embedded in transmembrane domains. Furthermore, transmembrane domain sorting signals may be important not only in the localization of single spanning membrane proteins, but may also determine the distributions of polytopic ion pumps such as the Na^+, K^+ and H^+, K^+-ATPases (see below). It should also be noted that the extracytoplasmic, or ecto domains of several apical proteins appear to incorporate directional signals. Roth et al. (273) have shown that the ectodomain of the influenza HA protein is sufficient to specify apical targeting. When a cDNA construct encoding an anchor-minus form of the HA protein, which lacks both the cytosolic and transmembrane segments, is expressed in polarized cells, it is secreted exclusively into the apical medium compartment. This is true as well for the polymeric immunoglobulin receptor (212). These results suggest that a signal involved in apical sorting resides in the lumenal portion of the HA molecule and that this signal remains interpretable when it is presented as a soluble protein or in association with portions of a basolateral membrane polypeptide. Finally, recent evidence suggests that N-linked sugar groups, which are also present on the extracytoplasmic domains of membrane proteins, can in some circumstances contribute apical sorting information (280). It is logical to conclude from this discussion that machinery necessary to read and interpret this putative ectodomain apical sorting information must be exposed at the lumenal surface of the organellar compartments involved in the segregation and targeting of newly synthesized membrane proteins.

As discussed above in the section on sorting pathways, not all of the plasma membrane proteins expressed by polarized epithelial cells pursue a direct course to their sites of ultimate functional residence. The polymeric immunoglobulin receptor (pIgR) for example, when examined in its native liver (129, 215, 311) or in transfected MDCK cells (214), travels first to the basolateral surface and subsequently to the apical pole. A number of studies have examined the contributions that various portions of pIgR molecule may make to this complicated sorting behavior. Anchor-minus ectodomain constructs of the pIgR are secreted apically from transfected MDCK cells (212). Furthermore, deletion of the pIgR cytosolic tail results in a membrane protein that travels directly to the apical surface without ever appearing at the basolateral side (213). These observations have led to the suggestion that the ectodomain of the pIgR receptor contains an apical sorting signal and that this protein's cytosolic tail embodies information that is required for its initial appearance at the basolateral plasma membrane. Extensive mutational analysis reveals that a trio of amino acids in the sequence his, arg, Val, is primarily responsible for the vectorial targeting of the newly synthesized pIgR protein to the basolateral plasmalemma. This motif constitutes yet another addition to the growing collection of distinct amino acid sequences that can encode basolateral sorting (8, 261).

During its tenure at the basolateral membrane, the pIgR's cytosolic tail becomes phosphorylated on a serine residue. The phosphorylation event occurs both in liver (159) and in transfected MDCK cells (44). Intriguing experiments demonstrated that the addition of this phosphate group acts as a switch that allows the apical sorting signal to predominate and results in the protein's transcytosis to the apical side. Site-directed mutagenesis has been performed on the cDNA encoding the pIgR in order to convert the serine of interest into either an alanine or an aspartate residue (44). When expressed in MDCK cells, the wildtype as well as the two mutant forms, are all initially targeted to the basolateral surface and all three undergo endocytosis and recycling at similar rates. Interestingly, however, while the wildtype receptor undergoes fairly rapid transcytosis, the alanine form remains largely associated with the basolateral plasma membrane. In contrast, the aspartate form is transcytosed at a rate that exceeds that characteristic of the nonmutant form. These observations suggest that the negative charge associated with the phosphate and aspartate residues permits or activates the incorporation of the pIgR into transcytotic vesicles and thus initiates the protein's delivery to the apical surface. The mechanism through which this signal is detected and interpreted remains unclear.

The recognition and segregation of pIgR destined for transcytosis probably occurs in an endosome following internalization from the basolateral surface. The second sorting event involved in the targeting of the pIgR is thus almost certainly completed at a subcellular location distinct from the TGN. This behavior suggests that, once again, the sorting of apical from basolateral proteins need not occur exclusively on

the exocytic pathway. The endosome or an endosome-related compartment appears competent to sense and act on the sorting signals that are necessary for the pIgR's apical localization. It remains to be determined whether signals detected in the endosome correspond to the same ectodomain-associated information that mediates the apical secretion of an anchor-minus form of the pIgR. The segregation of this secretory form to the apical pathway almost certainly occurs during its passage through the Golgi and is not likely to involve elements of the endocytic apparatus.

Most ion transport proteins and receptors span the membrane several times and many are composed of multiple subunits. Their intricate structures complicate the search for sorting signals and increase the likelihood that multiple independent or hierarchical signals might be present. This is clearly the case for the gastric H^+,K^+-ATPase. Acid secretion in the stomach is mediated by the gastric H^+,K^+-ATPase. This dimeric ion pump is stored within an intracellular population of membranous vesicles known as tubulovesicular elements (TVEs) in gastric parietal cells. Stimulation of acid secretion by secretagogues induces the TVEs to fuse with the parietal-cell apical plasma membrane, resulting in the formation of deeply invaginated secretory canaliculi rich in H^+,K^+-ATPase. The cessation of acid secretion involves the retrieval of the H^+,K^+-ATPase from the cell surface and the regeneration of the TVE storage compartment (342). Both the α and β subunits of the H^+,K^+-ATPase belong to the large P-type ATPase gene family (126). Their closest cousins in this collection are the corresponding α and β subunits of the Na^+,K^+-ATPase. Interestingly, while the H^+,K^+-ATPase functions at the apical surface of gastric parietal epithelial cells, the Na^+,K^+-ATPase is restricted in its distribution to the basolateral plasmalemma in this and most other epithelial cell types (40). The homology relating these ATPase functions has permitted the creation of chimeric ion pumps, whose subunits are composed of complementary portions of the H^+,K^+ and Na^+,K^+-ATPase α and β polypeptides. By expressing these constructs in cultured polarized epithelial cells it has been possible to determine the molecular domains of the ion-pump subunit proteins that are responsible for their sorting. Through this analysis it has become clear that both the α- and β-subunit polypeptides of the H^+,K^+-ATPase contain molecular signals that can contribute to the targeting of the holo-enzyme (101, 220). Expression of a large number of progressively more refined α-subunit chimeras reveals that an eight amino acid sequence within the α subunit of the H^+,K^+-ATPase is sufficient to specify apical sorting (68). This domain is predicted to reside within a transmembrane helix, thus suggesting that protein-lipid or protein–protein interactions within the plane of the membrane are responsible for pump sorting.

The β subunit of the H^+,K^+-ATPase contains a tyrosine-based sorting signal that functions to internalize the pump complex from the surface of the gastric parietal cell and return it to an intracellular regulated storage compartment (60, 101). This internalization is responsible for the cessation of gastric acid secretion following the removal of secretagogue stimulation. This was demonstrated by generating a transgenic mouse that expresses an H^+,K^+-ATPase β subunit lacking this endocytosis signal (60). These animals are unable to re-internalize H^+,K^+-ATPase from the apical surfaces of their gastric parietal cells. Consequently, they produce elevated gastric acid secretion during the interdigestive period. Mice carrying the mutant β-subunit develop gastritis and gastric ulcerations with histologic features that are essentially identical to those found in human disease. Examination of renal potassium clearance in these animals reveals that the same β-subunit sorting signal regulates active potassium resorption in the collecting tubule (338).

Several other studies have begun to define other signals employed in the polarized sorting of polytopic membrane proteins. Recently, for example, a novel motif has been identified in the cytoplasmic tail of rhodopsin, the seven-membrane span receptor that mediates this protein's apical sorting when it is expressed in MDCK cells (51, 316). Another member of the seven transmembrane G protein–coupled receptor family, the P2Y2 receptor, manifests an apical sorting signal in one of its extracellular loops (258). Furthermore, studies of neurotransmitter re-uptake systems have demonstrated that the four members of the highly homologous GABA transporter gene family are differentially sorted in epithelial cells and in neurons (3, 254). The GAT1 and GAT3 isoforms, which are restricted to axons when expressed endogenously or by transfection in neurons, are sorted to the apical membranes of epithelial cells. The GAT2 and betaine transporters, which are 50%–67% identical to GAT1 and GAT3, behave as basolateral proteins in epithelia and are restricted to dendrites when expressed in neurons. Production of chimeric and deletion constructs have permitted the identification of very short amino acid sequences at the extreme C-terminal tails of these transporters that manifest targeting information. The nature of these sequences suggests that they may interact with polypeptides containing PDZ-type protein–protein interaction domains, raising the possibility that this newly characterized association may play a direct role in the sorting of ion transport proteins (219). A similar PDZ-dependent mechanism also appears to mediate the apical trafficking of CFTR (50, 198, 216, 217).

Cell Type–Specific Sorting Patterns

The message encoded within a membrane protein's sorting signal is dependent not only on its own specific biochemical composition, but also the cellular context in which it is expressed. Several examples of membrane proteins that are differentially targeted in distinct epithelial cell types have been documented. The vacuolar H^+-ATPase, for example, accumulates at the apical surfaces of α-type intercalated cells but at the basolateral plasmalemmas of β-type intercalated

cells in the renal collecting duct (4). Similarly, the Na^+,K^+-ATPase is basolateral in most epithelia, but behaves as an apical protein in cells derived from the neural crest, such as the choroid plexus and retinal pigment epithelium (5, 113). Targeting of particular proteins or classes of proteins can also vary as a function of the differentiation states of epithelial cells. For example, the sorting of well-characterized polarity markers expressed in *Drosophila* via germ-line transformation was followed in the developing *Drosophila* embryo. Human placental alkaline phosphatase (PLAP) is a glycosylphosphatidyl inositol (GPI)–linked protein that accumulates at the apical membranes of mammalian epithelial cells. A chimeric construct composed of the transmembrane and cytosolic portions of the vesicular stomatitis virus (VSV) G protein coupled to the ectodomain of PLAP has been found to behave as a basolateral protein when expressed in the MDCK cell system (37). The subcellular distributions of these proteins were examined in the epithelial tissues of transgenic *Drosophila* embryos that expressed these proteins under the control of a heat shock promoter (287). In the surface ectoderm both PLAP and PLAPG were restricted to the basolateral membranes throughout development. Internal epithelia derived from the surface ectoderm accumulated PLAP at their apical surfaces, while PLAPG retained its basolateral distribution. The redistribution of PLAP from the basolateral to the apical plasma membrane was found to be coincident with the invagination of the surface epithelium to form internal structures, suggesting that the sorting pathways that function in the epithelium of the *Drosophila* embryo are developmentally regulated. More recent studies demonstrated that various lines of renal epithelial cells can interpret differently a specific, defined sorting motif. When expressed by itself in LLC-PK1, cells, the gastric H^+,K^+-ATPase β subunit accumulates at the apical plasmalemma. As noted previously, the amino acid sequence of the gastric H^+,K^+-ATPase β subunit reveals that its cytoplasmic tail contains a 4–amino-acid motif, YXRF, which has been shown to function as an endocytosis motif for the holoenzyme in gastric parietal cells in situ (54, 60, 96, 101). Since tyrosine-containing endocytosis motifs have been shown to be sufficient to ensure basolateral targeting of membrane proteins in MDCK cells (34, 135, 163, 192, 315), it is perhaps surprising that the H^+,K^+ β behaves as an apical protein in LLC-PK1 cells. To further examine the H^+,K^+-ATPase β-subunit's sorting signal, MDCK cells were stably transfected with the rabbit gastric H^+,K^+-ATPase β-subunit cDNA (274). Examination of this protein's distribution by surface immunofluorescence and cell surface biotinylation indicated that it was restricted to the basolateral plasma membrane.

Mutagenesis experiments support the hypothesis that sorting information is contained within the cytoplasmic tail and, more specifically, within the tyrosine-based sorting motif of the H^+,K^+-ATPase β-subunit. These data suggest that sorting and internalization motifs are, as a class, differentially interpreted in the MDCK and LLC-PK1 cell lines (251). A possible molecular basis for this sort of disparate behavior has recently emerged. While MDCK cells express the μ1b subunit of the AP1 adapter complex, this protein is not found in LLC-PK1 cells (242). As will be discussed in the next section on sorting mechanisms, it is now established that μ1b expression can ensure the basolateral targeting of membrane proteins bearing tyrosine-based sorting motifs (78). While this μ1b-dependent mechanism appears to be sufficient to account for the sorting behaviors of a number of proteins that are differentially sorted by MDCK and LLC-PK1 cells, it appears not to explain the distribution of the H^+,K^+-ATPase β subunit in these two cell types (67).

In light of both the multiplicity of sorting signals presented in the preceding section and the apparent potential for heterogeneity in their interpretation discussed above, it is natural to wonder whether any logic or consistency governs nature's solution to the deceptively simple problem of apportioning proteins among two separate membrane domains. Upon further reflection, however, the complexity and degeneracy of the "sorting code" can be seen as a tremendous virtue. Two different epithelial cell types may need to target a given membrane protein to opposite surfaces of their respective plasma membranes in order to fulfill their unique physiologic functions. These same functions may also require, however, that other membrane proteins occupy the same surface distributions in both cellular contexts. Thus, while the sodium pump occupies the apical membranes of the cells of the choroid plexus and the basolateral membranes of renal epithelial cells, receptors for basement membrane components are present at the basolateral surfaces of both cell types. If only a single class of basolateral sorting signal and a single class of apical sorting signal existed, then it would not be possible for a cell to selectively alter the distribution of one set of plasmalemmal proteins without simultaneously altering the distributions of the entire population of the plasma membrane. In order to target the sodium pump to the apical surface, choroid plexus epithelial cells would be forced to target basement membrane receptors there as well. This would obviously constitute a wasteful compromise. In order to endow each epithelial cell type with the capacity to select individualized complements of proteins for its apical and basolateral domains, a dizzying multitude of sorting signals has evolved. Cells can thus customize the distributions of proteins among their plasmalemmal domains without the constraints that would be imposed by a limited number of sorting signals. According to this interpretation, sorting signals do not specify a specific destination such as apical or basolateral. Instead, they specify classes of proteins whose members are always sorted together. The membrane domain to which any one of these classes is sorted will depend on the cellular context in which it is expressed, and will be determined by the idiosyncratic array of sorting machinery and pathways present in each individual epithelial cell type.

Sorting Mechanisms

It is safe to say that we are just beginning to understand the mechanisms through which the sorting signals discussed above exert their effects and ensure the polarized delivery of newly synthesized plasma-membrane proteins. The strong evidence for the existence of sorting signals leads quite naturally to the postulate that sorting receptors must exist that are capable both of recognizing these signals and of transducing their messages to the relevant cellular machinery. Such receptors have, in fact, been demonstrated in the case of lysosomal enzyme sorting. Targeting of a newly synthesized hydrolase to the lysosome is mediated by the interaction between the enzyme's mannose-6-phosphate (man-6-P) recognition marker and one of two receptors that bind man-6-P–bearing ligands in the Golgi and mediate their segregation to prelysosomal endosomes (155). Binding of newly synthesized lysosomal enzymes to the man-6-P receptors is pH dependent. At the relatively neutral pH of the Golgi, ligands are tightly bound, whereas in the acid environment of the prelysosomal endosome they are rapidly released. No such well-characterized receptor systems have yet emerged to explain the sorting behavior of secretory and membrane proteins in polarized cells. While sorting receptors for secretory proteins remain to be identified definitively, some progress has been made in understanding how such receptors might function. Lysosomotropic amines, such as NH_4C1 and chloroquine, elevate the lumenal pH of acidic organelles (194). The resulting neutralization of acidic compartments can have profound effects on sorting. In the case of lysosomal enzyme targeting, addition of NH_4Cl raises the pH of the prelysosomal endosome and thus prevents the acid-dependent unbinding of newly synthesized hydrolases from the man-6-P receptor (155). In the continued presence of the drug, the Golgi becomes depleted of receptors available to complex with free ligand. Newly synthesized enzymes bearing the man-6-P recognition marker are thus secreted constitutively and by default. Experiments on cultured polarized epithelial cells suggest that a similar pH-dependent mechanism may function in the sorting of basolateral secretory proteins (43).

Laminin and heparan sulfate proteoglycan are constituents of epithelial basement membranes (181, 200). Studies of permeable-filter–grown MDCK cells supports revealed that both of these proteins are normally secreted predominantly into the basolateral medium compartment (43). When secretion from cells treated with NH_4Cl was monitored, it was found that both proteins were released into both media compartments in roughly equal proportions. Removal of the drug reversed this effect and restored normal basolateral secretion. As mentioned above, studies have demonstrated that the secretory default pathway for MDCK cells—that is, the route pursued by soluble proteins that lack any means of interacting with the cellular sorting machinery—is apical and basolateral (43, 103, 153). It appears, therefore, that targeting of these two basolateral secretory proteins requires the participation of an intracellular acidic compartment. Elevation of the lumenal pH of this compartment reversibly blocks laminin and HSPG (heparan sulfate proteoglycans) sorting and results in their apical and basolateral default secretion.

Although the nature of the dependence of this sorting event on acidic compartments remains unknown, it is interesting to speculate that a mechanism similar to that which functions in lysosomal enzyme sorting may also be involved in routing basolateral secretory proteins. In such a model, binding or unbinding of laminin and HSPG from a sorting receptor would require the participation of an acidic organellar pH. Confirmation of this hypothesis will await the identification of such a pH-dependent–binding protein with affinity for these and other basolaterally targeted proteins (52). Finally, it is worth noting that the basolateral sorting of the Na^+,K^+-ATPase and the apical sorting of the influenza HA protein and a complex of secretory polypeptides occur normally in the presence and absence of NH_4C1 (41, 43, 183). It would appear, therefore, that different mechanisms are brought to bear in ushering different classes of proteins to their sites of ultimate functional residence.

Tyrosine-Based Motifs and Adapters

Recent studies suggest that several different classes of soluble proteins may regulate the subcellular distributions of proteins bearing tyrosine-based signals. Perhaps the best understood of these are the adaptins (252). The adaptins comprise a group of peripheral membrane proteins that mediate the interaction between transmembrane proteins and the clathrin skeletons of coated pits and vesicles. Adaptins recognize and bind to tyrosine-containing coated pit localization sequences and link the proteins bearing these motifs to the clathrin coat (21, 241, 250-252). Adaptins can thus be considered to be among the most proximal elements of the endocytic sorting machinery—they recognize polypeptides endowed with endocytosis signals and ensure that they are incorporated into the specified internalization pathway. Distinct classes of adaptins function in the segregation of proteins into the coated structures associated with the trans-Golgi network and into cell surface coated pits (252). While AP2 adapters mediate internalization of proteins from the cell surface, AP1 adapter complexes participate in trafficking proteins out of the TGN. The μ subunits of adapter complexes appear to be responsible for interacting with tyrosine-based motifs (241). Two isoforms of μ subunits are found in AP-1 complexes. The μ1a protein is ubiquitously expressed and is found in both polarized and nonpolar cell types. The μ1b protein is instead found in only a subset of polarized cell types (242). As noted above, proteins bearing tyrosine-based motifs are basolaterally sorted in MDCK cells but accumulate apically in LLC-PK1 cells (274). It was noted that MDCK cells express μ1b, whereas this protein is absent from LLC-PK1 cells. Remarkably, expression of μ1b in LLC-PK1 cells at least partially "normalizes" their sorting properties, so that

many (but not all) membrane proteins containing tyrosine-based signals are directed to the basolateral surface (78). Thus, μ1b constitutes perhaps the best characterized component of the sorting machinery. It is clearly capable of recognizing a class of sorting signals and acting on the instructions that they convey.

It is interesting to note that recent studies demonstrate that different proteins bind to and interpret the messages encoded by tyrosine-based and di-leucine endocytosis motifs. Over expression of tyrosine-motif–containing proteins can inhibit the endocytosis of other proteins carrying a similar endocytosis signal, presumably by competing for limited quantities of the adapter proteins that cluster proteins bearing these signals into clathrin-coated pits. This intervention does not affect, however, the internalization of proteins endowed with di-leucine motifs, indicating that they must be recognized and interpreted by a different class of polypeptides (180). It appears that the β subunits of adapter complexes interact with di-leucine motifs (260). Finally, a very different type of protein has been shown to interact with a tyrosine-based proline-rich sequence in the C-terminal tails of epithelial sodium channel (ENaC) subunits. The Nedd-4 protein possesses an ubiquitin ligase domain, and through its interaction with the ENaC tails may lead to downregulation of these channels through degradation (300).

The association of basolateral membrane proteins such as the Na^+,K^+-ATPase with elements of the subcortical cytoskeleton (211, 229) has led to the speculation that this interaction may play a role in targeting. Evidence in support of this proposition was found in the studies, described above, which suggested that, at least in one MDCK cell clone, the Na^+,K^+-ATPase may be delivered in equal proportions to the apical and basolateral surface (117). Apically delivered material may be rapidly degraded, whereas the basolateral sodium pump would be stabilized through interaction with the cytoskeleton and consequently turn over very slowly. The pump's polarized distribution would thus be the product of differential susceptibility to degradation rather than sorting at the level of the Golgi. The degree to which stabilization through interaction with the cytoskeleton contributes to the polarized distribution of the sodium pump or any other proteins remains to be established.

The observation that all of the glycolipids and GPI-linked proteins associated with epithelial cells tend to be found in the apical plasmalemmal domain has led to the proposal that lipids may play a role in membrane protein sorting (294). Since glycolipids and GPI-linked proteins are only associated with the outer leaflet of the plasma membrane, these molecules will be exposed at the lumenal face of the organelles of the biosynthetic pathway. Any sorting machinery that interacts with glycolipids, therefore, must do so either at the lumenal surface or within the plane of the membrane itself. These constraints have suggested to some investigators the possibility that lipid–lipid interactions are sufficient to segregate apically directed glycolipids and GPI-linked proteins into distinct patches during their residence in the Golgi. These self-assembled patches could then serve as the nuclei from which apically directed vesicles would bud. The biophysical properties of these patches might be involved in ensnaring other apically directed proteins as well as the components necessary to appropriately target the resultant transit vesicle (295). While evidence of lipid patches exists in both in vitro and in vivo systems (10, 327), their precise role in the sorting process remains to be elucidated. Independent of its applicability, however, this model is extremely interesting. It is a useful reminder that forces other than simple receptor–ligand interactions are likely to be involved in generating and maintaining the anisotropic protein distributions that define the polarized state.

As noted above, several proteins are targeted to the apical membrane by virtue of signals embedded within their transmembrane domains. The fact that the amino acid residues of a transmembrane domain may be in direct contact with lipid molecules suggests the possibility that they may mediate apical sorting through interactions with glycosphingolipid-rich membrane domains. According to this hypothesis, the composition of its transmembrane domain may permit a protein to partition into glycosphingolipid-rich patches and thus to become concentrated in a region of the membrane that will give rise to an apically directed transport vesicle. GPI-linked proteins that have become associated with glycosphingolipid-rich membrane domains are insoluble in 1% Triton X-100 at 4°C. When a cell lysate prepared in this fashion is fractionated on a sucrose gradient, insoluble proteins are found near the top of the gradient, whereas soluble proteins remain in the heavier fractions (10). Interestingly, the transmembrane domain of the apical protein influenza neuraminidase carries apical sorting information and also enables the protein to incorporate into insoluble.

Glycosphingolipid-Rich Membrane Domains

Evidence suggests that the basolateral sorting of the Na^+,K^+-ATPase might occur as the result of exclusion from glycosphingolipid-rich membrane regions. MDCK cells treated with the drug fumonisin, which prevents sphingolipid synthesis, randomly deliver newly synthesized Na^+,K^+-ATPase to both cell surface domains. In light of the observation that H^+,K^+ and Na^+,K^+-ATPase targeting appears to be mediated by a transmembrane sequence, it is tempting to hypothesize that their differential distributions are determined by their differing abilities to partition into glycosphingolipid-rich membrane regions. This possibility has been examined by determining the detergent solubility of apically-directed pump chimeras. When epithelial cells expressing an apically targeted pump chimera are lysed on ice with 1% Triton X-100, the endogenous GPI-linked alkaline phosphatase migrates to the top of a sucrose floatation gradient. The endogenous Na^+,K^+-

ATPase on the other hand, appears in the heavier fractions, a pattern typical for detergent-soluble membrane proteins. If the chimera containing the fourth transmembrane span of the gastric H^+,K^+-ATPase partitions into insoluble glycolipid patches, it should codistribute with the GPI-linked alkaline phosphatase. However, the chimera is found in the same fractions as the Na^+,K^+-ATPase, and is completely absent from the fractions containing alkaline phosphatase activity. Thus, an apically located chimera containing the fourth transmembrane domain of the H^+,K^+-ATPase exhibits no difference in its detergent solubility characteristics as compared with the basolaterally located Na^+,K^+-ATPase. This result suggests that mechanisms other than lipid association may be responsible for the sorting function of at least one transmembrane domain localization signal.

As discussed above, the C-terminus of GABA transporter GAT-3 appears to be important for its apical localization in MDCK cells (219). The final residues of this C-terminal tail, threonine, histidine, and phenylalanine (THF), are reminiscent of the sequences present at the extreme C-terminal tails of proteins known to associate with members of the membrane associated guanylate kinase (MAGUK) family. The MAGUK proteins incorporate one or more copies of the PDZ domain, which is named for three of the proteins in which the sequence homology defining this protein–protein interaction motif were first identified: PSD-95/SAP90, Dlg, and ZO1. Interactions between the PDZ domain of a MAGUK protein and the extreme cytoplasmic tail of an integral membrane polypeptide appear to be important in organizing the surface distributions of intrinsic membrane proteins (71, 297).

Observations obtained from a number of experimental systems provide further evidence for the involvement of PDZ domain–containing polypeptides in epithelial membrane protein sorting (148). The LET23 receptor tyrosine kinase is localized to the basolateral cell surfaces of vulvar epithelial cells in *C. elegans*. Genetic studies reveal that at least three proteins contribute to the generation or maintenance of this distribution. Mutation of the lin2, lin7, or lin10 genes leads to loss of LET-23 basolateral polarity. Each of the proteins encoded by these genes includes one or more PDZ domains. A mutation in the *Drosophila* discs lost protein, which contains multiple PDZ domains, also leads to the mis-localization of several apical and basolateral proteins in the epithelial structures of affected embryos (24). It would appear, therefore, that PDZ domain–containing proteins may play a direct role in the polarized sorting of at least some membrane proteins or may be required for the generation or definition of polarized domains. These observations may be especially relevant to physiologic function of polarized renal epithelial cells, since a number of important ion transport proteins, including CFTR and NHE3, appear to interact with cytoplasmic proteins containing PDZ domains (115, 288, 337). It seems likely that these interactions may play a role in establishing these proteins' distributions and hence determining their capacity to participate in vectorial ion transport.

Finally, it is important to note that once proteins have been sorted into the vesicles that will carry them to the appropriate cell surface domain, these vesicles need to themselves be targeted appropriately. Presumably, the vesicular membranes include proteins that ensure that the vesicles will interact and fuse with only the proper domain of the epithelial plasmalemma. This recognition machinery is likely to include components of the membrane fusion machinery, such as vesicular SNARE (soluble NSF attachment receptor) proteins (76). SNARE proteins present in both vesicular and target membranes form complexes that appear to be necessary for most normal cellular fusion processes. The extent to which different members of the SNARE family impart specificity to intracellular vesicular fusion events remains to be established (140, 141, 175). Interestingly, however, a newly identified component of the machinery involved in vesicular targeting in yeast has recently been identified in mammalian cells (114). This Sec 6/8 complex appears to play a role specifically in the fusion of basolaterally directed, but not apically directed post-Golgi carrier vesicles in epithelial cells (98). It is likely that the number of "destination-specific" vesicular and plasma membrane proteins important for directing vesicular traffic in polarized cells will continue to grow.

EPITHELIAL CELL POLARITY AND RENAL DISEASE

Because kidney function is dependent on the polarity of tubular epithelial cells, any condition that compromises this polarity will lead to renal failure (77, 301). In general, this may occur through neoplastic processes, cell injury due to ischemia or nephrotoxicity, or inherited genetic effects (77, 282). Each of these may affect the tubular epithelial cells, their surrounding environment including the basal lamina and interstitial compartment, or both.

Carcinogenesis

During neoplastic growth it can be appreciated on the basis of morphology alone that the changes in cell and tissue organization wrought by tumorigenesis are likely to affect cell polarity (282). Model studies confirm this suspicion. When MDCK cells, which are not normally tumorigenic, are oncogenically transformed by introduction of the v-Ki-*ras* oncogene, they are converted from a simple epithelium to a multilayer, with great heterogeneity in overall cell morphology (282). Ultrastructural examination of these cells suggests that apical–basal polarity is severely compromised (282). Microvilli are diminished from the cells at the top layer, and organization of the cytoplasm is scrambled. Golgi complexes and centrosomes, which normally reside in an apical supranuclear location, are now

found randomly positioned (282). Despite this apparent high degree of disorganization, immunocytochemical localization of specific antigens and physiologic measurements suggests that polarity is not totally disrupted. Basolateral proteins, including Na^+,K^+-ATPase and the cell-adhesion molecule E-cadherin, are restricted to regions of cell–cell contact, as in normal polarized MDCK cells (282). Apical proteins, on the other hand, are randomly localized to the free surface of the multilayered epithelial as well as to areas of cell–cell contact in cells throughout the multilayer (282). The tight junctional antigen ZO-1 is found typically at the point where the free and adherent surfaces of the uppermost cell layer meet as well as at a number of sites within the multilayer (282). The latter may be intercellular lumina or canaliculi connected to the upper surface. This localization probably reflects the presence of functional tight junctions, because the multilayer is both electrically tight and impermeable to inulin. Disruption of polarity of this type might have significant implications for net ion flow. For example, redistribution of a sodium channel normally found on the apical surface to both the apical and basolateral domains might occur as a result of the oncogenic process (77). If tight junctions remain intact and the Na^+,K^+-ATPase is retained on the basolateral surface under these circumstances, then sodium transport would be short-circuited, making it much less efficient. It is also interesting to note that recent studies demonstrate that proteins encoded by tumor suppressor genes may function as key regulators of polarity. Mutations in the gene encoding the LKB1 protein kinase are responsible for Peutz–Jaeger syndrome, an inherited form of tumor susceptibility associated with the development of numerous hamartomas. As mentioned previously, epithelial cells expressing LKB1 that is constitutively activated are able to form polarized domains in the absence of cell–cell and cell–substratum contact (12). Thus, proteins that participate in epithelial polarization may function as tumor suppressors by virtue of their capacity to control the growth and morphogenesis of the cells in which they are expressed.

Ischemic Injury

Other alterations in cell polarity may come about through the effect of renal ischemia on the tubular epithelium (69, 243, 244, 296, 313). Ischemic episodes of less than 1 hour often do not lead to tubular necrosis but may, nevertheless, cause diminished sodium and water uptake by the proximal tubule (204). Such brief ischemia compromises the polarity of tubular cells, resulting in the redistribution of a fraction of the Na^+,K^+-ATPase from the basolateral domain to the apical domain, preventing net sodium uptake by the tubule (204, 298). At the same time, leucine aminopeptidase moves from the apical to the basolateral domain and also becomes intracellular, presumably through endocytosis. At later times, Na^+,K^+-ATPase and leucine aminopeptidase are randomly distributed on the plasma membrane of tubular epithelial cells remaining attached to the basement membrane or exfoliated into the lumenal space. The mechanism leading to this loss of polarity is not known. It is possible that ischemia, which is known to affect mitochondria and other organelles and to possibly alter the permeability of the plasma membrane, may result in increased cytoplasmic calcium concentrations (69, 296, 313). This, in turn, could disrupt elements of the cytoskeleton, perhaps affecting the maintenance of polarity. In fact, tubular epithelial cells have been observed by electron microscopy to develop basal densities following ischemia (296). These may represent disruption or perturbation of the cortical actin cytoskeleton. In support of this, in vitro studies with renal epithelial cell lines demonstrate that ATP depletion causes redistribution of actin from its normal locations in the cell cortex, terminal web, and microvilli to perinuclear cytoplasmic aggregates. Such alterations might affect transduction of spatial signals from the extracellular matrix to the polarization machinery along the lines previously discussed.

During reperfusion following renal ischemia, tubular epithelial cells detach from the basement membrane and accumulate in the lumen. It has been postulated that ischemia-induced depolarization of integrins from basal to apical domains of the plasma membrane contributes not only to cell detachment but also to cell aggregation and tubular obstruction. According to this hypothesis, at early times postischemia, redistribution of integrins would loosen attachment of cells from the basal lamina, allowing some of them to detach (91, 98, 234). Released cells would then aggregate and adhere to remaining tubular epithelial cells via their integrins. These would either bind directly to each other by homotypic interactions, or associate through bridging matrix molecules. Collections of such aggregates would obstruct the tubules, causing oliguria and destruction of renal tissue (91, 98, 234). In support of this hypothesis, integrins were observed to redistribute apically in oxidatively injured epithelial cell lines (91). Even more compelling was the observation that infusion of RGD peptides, which block some integrin-matrix interactions, appeared to ameliorate the effects of ischemia induced by clamping of the renal artery (233, 234). Recent in vivo findings using a rat model of renal ischemia do not, however, support this hypothesis, at least with regard to β1 integrins (355). Soon after reperfusion, β1 integrins were redistributed from a strictly basal to basolateral location in cells of the S3 segment of the proximal tubule, but did not appear on the apical plasma membrane at this time (355). Surprisingly, β1 integrins could not be detected by immunofluorescence in cells released from the basal lamina into the tubular lumen, precluding the possibility that they were mediating either cell aggregation or attachment of exfoliated cells to the residual tubular epithelium. Apical β1 integrins only appeared at later times postischemia as cells lost polarity in the process of regeneration (355).

Polycystic Kidney Disease

The progressive formation of renal cysts, which characterizes autosomal dominant polycystic kidney disease (ADPKD), has also been suggested to occur as a result of polarity defects. ADPKD is the most common potentially lethal dominant genetic human disease. Approximately 85% of all cases are linked to mutations in the PKD1 gene with another 10% linked to PKD2 (321). While the specific functions of the proteins encoded by these genes are the focus of intense study, the behavior of cyst epithelial cells in situ and in culture is consistent with a role for the PKD proteins in directing epithelial differentiation. Whereas renal tubular epithelial cells normally mediate fluid and electrolyte absorption, cyst epithelial cells carry out net secretion (105, 307). It has been suggested that the proximal cause of renal cyst formation in polycystic kidney disease may be the mis-targeting of Na^+,K^+-ATPase to the apical plasmalemma. According to this model, the presence of sodium pump at the apical surface leads to active apical ion secretion and the accumulation of lumenal cyst fluid (11, 341). Other studies suggest that mislocalization of Na^+,K^+-ATPase can not be the primary driving force for cyst fluid accumulation. When examined in cyst cells in culture or in situ, the Na^+,K^+-ATPase was found to be exclusively basolateral (36). Instead, the secretion appears to be driven by intracellular chloride accumulation via a basolateral $Na^+,K^+,2C1^-$ cotransporter and apical chloride exit through the CFTR protein (36). A similar mechanism is responsible for fluid secretion by the poorly differentiated epithelial cells lining the crypts of the small intestine. As these crypt cells migrate up the intestinal villus they mature functionally, metamorphosing from secretory into resorptive epithelial cells (82). It has been suggested that the secretory phenotype is characteristic of immature epithelial cells, while more highly developed epithelial cells acquire the capacity to absorb fluid and electrolytes (306). The physiologic similarities relating cyst and crypt epithelial cells has prompted the hypothesis that loss of appropriate PKD function results in the dedifferentiation of mature resorptive renal tubular epithelial cells into more primitive secretory cells. The precise mechanisms through the PKD1 and PKD2 mutations produce the dramatic pathology associated with ADPKD, and the potential role of epithelial differentiation and sorting pathways, remain to be determined.

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CHAPTER 2

Mechanisms of Ion Transport Across Cell Membranes and Epithelia

Luis Reuss
Texas Tech University Health Sciences Center, Lubbock, Texas, USA

INTRODUCTION

Ion transport by cell membranes serves two large purposes in pluricellular organisms, the maintenance of the volume and composition of the intracellular fluid and the preservation and regulation of the volume and composition of the extracellular fluid. The first process involves fluxes between the cell interior and its surrounding medium ("homocellular transport," 92), whereas the second one occurs because of transport across epithelial and endothelial cell layers (transcellular or "heterocellular" transport, 92). In addition, ion transport across intracellular membranes, which surround the nucleus and cytoplasmic organelles, are essential to generate and maintain ion concentration gradients between those organelles and the cytosol.

Needless to say, the narrowly regulated volume and ionic composition—inorganic cations (Na^+, K^+, H^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, phosphate, bicarbonate)—is essential for cell survival, and for the cell's normal functions. A similar argument can be made for the extracellular fluid compartments, that is, whole-body balances of water and the ions listed above are essential for the survival, growth, and development of the organism.

Our main focus in this chapter will be on the molecular mechanisms of ion transport by the plasma membranes of cells. The cell membrane is a phospholipid bilayer doped with abundant proteins. This structure is both a barrier between the cytoplasm and the extracellular fluid and the pathway for ion and water transport between the two compartments. For most ions, the lipid bilayer is the barrier and membrane transport proteins are the pathway for these fluxes.

The Cell Interior and Extracellular Fluid Have Different Ionic Compositions

A crucial property of living cells is their capacity to maintain an internal (intracellular or cytosolic) composition different from that of the surrounding (extracellular) medium. As all other ionic solutions, the cytosol and the extracellular fluid obey the principle of macroscopic (or bulk) electroneutrality, that is, the sum of cationic and anionic charges are the same in each compartment. As discussed below, there is a microscopic deviation from this principle at the membrane surfaces when there is a difference in electrical potential across the membrane, but the actual difference in ion concentrations is extremely small.

The maintenance of ionic asymmetry between intracellular and extracellular compartments is based on the existence of the cell membrane (or plasma membrane), which separates the cell interior from its surroundings. As shown schematically in Fig. 1, the membrane is a phospholipid bilayer ~4 nm thick, with high protein content. Membrane proteins can be tightly bound to the phospholipid bilayer (integral proteins, some of which span the membrane, known as transmembrane proteins) or can be loosely associated with the membrane surface (peripheral proteins). Transmembrane proteins perform many functions, including translocation of ions, nonelectrolytes, and water across the membrane (transport function, the main theme of this chapter); sensing and early transduction of extracellular events (signaling function); and attachment to components of the extracellular matrix or to adjacent cells (adhesion function).

Two properties of the cell membrane have been demonstrated to generate and maintain the intracellular ion composition essential for life: the barrier function and the transport function. This distinction is didactically convenient, although both functions are clearly linked. By the barrier function, the cell membrane prevents the flux of certain molecules; by the transport function, it translocates certain molecules. These two functions bring about a steady state in which cell volume and composition are kept constant and appropriate for cell survival. Relative to the extracellular fluid, some substances are maintained at high concentrations (e.g., K^+ and ATP), whereas others are maintained at low concentrations (e.g., Ca^{2+} and Cl^-) inside the cell.

The cell interior is not homogeneous, but rather a complex medium including a highly structured cytoplasm (cytosol and cytoskeleton) and numerous organelles. The latter are separated from the cytosol by their own membranes. Exchanges between each organelle and the cytosol occur in ways similar to those described for the plasma membrane

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