# Phosphorylation of the tight-junction protein ZO-1 in two strains of Madin–Darby canine kidney cells which differ in transepithelial resistance

Bruce R. STEVENSON,*§ James Melvin ANDERSON,† I. Deborah BRAUN* and Mark S. MOOSEKER*‡
*Department of Biology, Yale University, New Haven, CT 06511, †Department of Internal Medicine and Liver Study Unit, Yale School of Medicine, and ‡Department of Cell Biology, Yale School of Medicine, New Haven, CT, U.S.A.

A comparison was made of the phosphate content of the tight-junction-specific protein ZO-1 in two strains of Madin–Darby canine kidney cells which differ in transepithelial resistance, a parameter reflective of tight-junctional permeability. Analysis revealed that the ZO-1 from the low-resistance strain contained approximately twice as much phosphate as that from the high-resistance strain.

## INTRODUCTION

Diffusion of substrates through the epithelial paracellular pathway is limited by the tight junction (zonula occludens), a selectively permeable intercellular barrier which encircles each cell. It has been well-documented that tight-junctional permeability is under cellular control (see [1,2,3] for review); however, the specific constituents responsible for this control remained unidentified. A hypothesis has been advanced by Claude Goodenough [4,5] that junctional permeability is inversely proportional to the number of tight-junctional fibrils seen along the apical–basolateral axis of freeze-fractured epithelial cells. The identity and nature of the molecule(s) comprising these fibrils are unknown, although they are likely to be proteinaceous [3,6]. It has been clearly demonstrated that the cytoskeleton, specifically actin filaments, plays a role in the control of junctional permeability [7–13]; however, it is not known with what actin interacts at the tight junction.

Only relatively recently have the molecular components of the tight junction begun to be elucidated. ZO-1, the first element to be identified, is a high-molecular-mass (~225 kDa) protein associated with the tight junctions of a variety of epithelia and endothelia [14]. Physical analysis reveals ZO-1 to be an elongated monomeric protein that is peripherally associated with the junctional membrane and phosphorylated at serine residues [15]. An additional tight-junction-associated polypeptide, called 'cingulin', has been identified and also found to be a peripheral membrane protein localized in a variety of epithelial cell types [16,17]. ZO-1 and cingulin are distinct polypeptides with similar, but not identical, localizations [18].

In an effort to understand the role that ZO-1 plays in the physiology of the tight junction, a variety of junctional characteristics were compared in two strains of the Madin–Darby canine kidney (MDCK) cells which show a vast difference in transepithelial resistance [19]. Because the resistances of the plasma membranes are, in most cases, relatively high, this parameter is a measure of current flowing through the paracellular pathway and hence tight-junction permeability [20–22]. It was found that there is no difference in these cells in ZO-1 localization, the amount of ZO-1/$\mu$m of junction, or in overall junctional ultrastructure, including the number and branching complexity of fibrils seen in freeze–fracture [19]. This latter result is clearly contrary to the Claude & Goodenough hypothesis [4,5] and indicates that there are additional factors, perhaps involving molecular characteristics of junctional components, which need to be considered in an evaluation of junctional physiology. Here we report that one of these characteristics, the phosphate content of the ZO-1 found in these two strains of cells, is in fact different, although the functional import of this finding has not yet been explored.

## MATERIALS AND METHODS

MDCK strain I and II cells were grown to confluence on 24 mm Costar polycarbonate filter inserts, and the transepithelial resistance determined as described previously [14,19]. Mean resistances for the two strains were as follows: MDCK I, $4350 \pm 1864\ \Omega \cdot cm^2$ (mean ± S.D., $n = 31$); MDCK II, $55 \pm 29\ \Omega \cdot cm^2$ ($n = 35$). Duplicate filters from each strain of cells were placed in a six-well tissue-culture dish, rinsed once with phosphate-free Dulbecco modified Eagle medium (DMEM) supplemented with dialysed 10% fetal-bovine serum, and then incubated for 2 × 20 min in the same medium to deplete cells of phosphate. Labelling was done in the phosphate-free DMEM/10% fetal-bovine serum containing ~25 $\mu$Ci of [$^{32}$P]orthophosphate (carrier-free, 285 Ci/mg)/ml for various time periods. The labelling medium was discarded, and the filters rinsed once with cold Tris-buffered saline (TBS) before solubilizing total cell protein with 6 M-urea/0.1% Triton X-100/1 mM-dithiothreitol/5 mM-$MgCl_2$/5 mM-EGTA/150 mM-

Abbreviations used: MDCK, Madin–Darby canine kidney; DMEM, Dulbecco modified Eagle medium; TBS, Tris-buffer saline (10 mM-Tris/HCl/0.9% NaCl, pH 8.0).

§ Present address and address for correspondence and reprint requests: Department of Anatomy and Cell Biology, Faculty of Medicine, 5–14 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

NaCl/0.2 mM-phenylmethanesulphonyl fluoride/10 mM-Tris, pH 8.0. ZO-1 was immunoprecipitated from the solubilized protein and eluted into gel sample buffer as described [15]. Immunoaffinity-purified ZO-1 was electrophoresed on a 7.5%-(w/v)-polyacrylamide gel [23], transferred to nitrocellulose [24] and allowed to react with affinity-purified anti-(ZO-1 fusion protein) polyclonal antiserum [25] diluted to 20 $\mu$g/ml in TBS/BLOTTO (5% non-fat dried milk) [26] followed by $^{125}$I-protein A (~2 $\mu$Ci/ml; 92.3 $\mu$Ci/$\mu$g) in TBS/BLOTTO. The blot was dried, and an autoradiograph produced to overlay and mark the ZO-1 bands for excision from the nitrocellulose. The bands were then immersed in a liquid scintillant and counted for both $^{32}$P and $^{125}$I radioactivity, using counting-chamber limits such that no significant overlap between isotopic emission occurred. The ratio of these radioactivity counts ($^{32}$P/$^{125}$I) represents the specific activity of phosphate on ZO-1. Whole liver homogenate standards [6] were also run on the gel immunoblot to ensure protein A binding was in the linear range.

Similarly labelled duplicate filters were cut directly from their plastic chamber and analysed for total phosphate by the method of Duck-Chong [27]. The specific activity of total phosphate was determined by counting an aliquot of the ashed and solubilized filter. The assumption was made that this activity reflects the rapidly-turning-over ATP pool responsible for labelling ZO-1. $^{32}$P/total cell phosphate versus time was plotted for each strain, and the curves were fitted by non-linear regression analysis using the ENZFIT fitting program (Elsevier). The ratio between the two curves was determined for every time point, and the $^{32}$P/$^{125}$I (ZO-1) for the strain-I cells was adjusted according to these ratios. The ZO-1 phosphate specific activity of strain II was then plotted and the best-fit curve determined by non-linear regression. The curve was then scaled so that the curve limit was unity. The same scaling factor was applied to the total-phosphate-specific-activity-adjusted strain I values and these plotted on the same graph. All the values from four separate experiments are presented. Determination of total phosphate specific activity was performed twice. The $t_{\frac{1}{2}}$ of phosphate incorporation for each strain was calculated from the standard first-order rate equation:

$$[A] = [A]_0 e^{-kt}.$$

For the $t_{\frac{1}{2}}$ of phosphate incorporation:

$$[A]/[A]_0 = \tfrac{1}{2} = e^{-kt_{\frac{1}{2}}}$$

$$\ln \tfrac{1}{2} = -kt_{\frac{1}{2}}$$

or

$$t_{\frac{1}{2}} = 0.693/k$$

where $k$ is the rate constant obtained from the fitting program.

## RESULTS

Confluent monolayers of MDCK strains I and II were labelled for increasing periods of time with [$^{32}$P]ortho phosphate, and the amount of label on ZO-1 in each was determined by immunoprecipitation and isotope quantification (see the Materials and methods section). The results of this investigation are shown in Fig. 1. The rate of incorporation of phosphate into ZO-1 of each strain was the same, with a $t_{\frac{1}{2}}$ of approx. 3 h. This incorporation reached saturation levels within approx. 12 h and remained at a steady state for at least another 12 h.

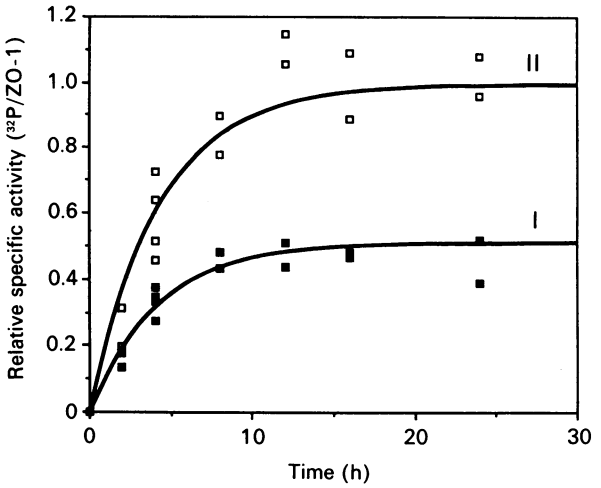


**Fig. 1. Analysis of the specific activity of phosphate on ZO-1**

Filter-grown MDCK cells from both strains were metabolically labelled with [$^{32}$P]orthophosphate for increasing time periods, the ZO-1 immunoprecipitated from total solubilized cell protein, and the ratio $^{32}$P/total ZO-1 proein determined. These ratios from MDCK I and II monolayers are plotted relative to each other, with the MDCK II data scaled to unity. The specific activity of total cell phosphate, a reflection of the activity of the ATP labelling pool, was also determined. The ratio of the difference in total phosphate specific activity for each time point was used to adjust the ZO-1 phosphate activity values. The graph shows data from four separate experiments. Best-fit curves to a single-exponential kinetic equation were determined by non-linear regression analysis. ■, MDCK I; □, MDCK II. The ratio (I/II) of the curve saturation limits is 0.51 ± 0.08, indicating that ZO-1 from the MDCK II cells contains approximately twice as much phosphate as MDCK I ZO-1. Phosphate was incorporated into the two cell strains at approximately the same rate ($t_{\frac{1}{2}, \text{MDCK I}}$ 2.9 h; $t_{\frac{1}{2}, \text{MDCK II}}$ 3.0 h. The two strains differ predominantly in the steady state level of phosphate incorporated.

In contrast with the similarity in the kinetics of $^{32}$P incorporation into ZO-1, the actual plateau levels of ZO-1 phosphate specific activity for the two cell strains were significantly different. The steady-state limit values had a ratio (MDCK I/MDCK II) of 0.51 ± 0.08, indicating that the ZO-1 of the low-resistance strain-II cells contained twice as much phosphate as that of the high-resistance strain-I cells.

## DISCUSSION

Previous examination of tight-junction structure and ZO-1 distribution and content in these two strains of MDCK cells [19] indicated that the observed difference in transepithelial resistance, and presumably tight-junctional permeability, might be related to the biochemical properties of individual junctional elements involving a putative channel and/or peripheral junction components. Phosphorylation is one obvious means by which the cell regulates protein function, and there are considerable data indicating that pharmacological

manipulation of various kinase systems can affect the permeability properties of the tight junction [28–31]. The studies described here indicated that the ZO-1 in the two strains of cells contained different amounts of phosphate (Fig. 1). The following caveats should be considered in the interpretation of these results. A direct comparison of the specific activities of the ATP labelling pool in the two strains of cells was not possible. Because ATP has a rapid turnover, we assumed that its specific activity is reflected by the specific activity of total cellular phosphate. MDCK I and II contain identical total amounts of phosphate; however, the kinetics of incorporation of $^{32}P$ into those pools are different in each strain (results not shown). Although the specific activities must, by definition, eventually reach equivalence, we adjusted the ZO-1 phosphate specific activities to reflect this difference in incorporation rates. The ratio (MDCK I/MDCK II) of steady-state limits in the adjusted curve is $0.51 \pm 0.08$. In the absence of adjustment for total phosphate specific activity the ratio (I/II) would be approx. 0.40. It also should be emphasized that this observed difference in ZO-1 phosphate content does not demonstrate a direct correlation between junctional permeability and ZO-1 phosphorylation. These results do show, however, that tight junctions which are indistinguishable by structural criteria [18] can differ in the molecular properties of junctional components.

We are grateful to Jon Morrow for his extensive help and advice, Deb Sliker for her technical assistance, Tom Coleman for his help with the graphics, and Michelle Peterson for her continuing grammar lessons. The work presented here was supported by a grant from the National Institutes of Health to M.S.M. (GM37556) and a Program Project Grant (TO-1-KD38979, to M. Kashgarian, Principal Investigator). J.M.A. is supported by a Terry Kirgo Memorial Fellowship from the American Liver Foundation and a Lucille P. Markey Charitable Trust Award.

## REFERENCES

1. Gumbiner, B. (1987) Am. J. Physiol. **253**, C749–C758
2. Madara, J. L. (1988) Cell (Cambridge, Mass.) **53**, 497–498
3. Stevenson, B. R., Anderson, J. M & Bullivant, S. (1988) Mol. Cell. Biochem. **83**, 129–145
4. Claude, P. & Goodenough, D. A. (1973) J. Cell Biol. **58**, 390–400
5. Claude, P. (1978) J. Membr. Biol. **39**, 219–232
6. Stevenson, B. R. & Goodenough, D. A. (1984) J. Cell Biol. **98**, 1209–1221
7. Bentzel, C. J., Hainau, B., Edelman, A., Anagnostopulos, T. & Benedetti, E. L. (1976) Nature (London) **264**, 666–668
8. Elias, E., Hruban, Z., Wade, J. B. & Boyer, J. L. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2229–2233
9. Meza, I., Ibarra, G., Sabanero, M., Martinez-Palomo, A. & Cereijido, M. (1980) J. Cell Biol. **87**, 746–754
10. Madara, J. L., Barenberg, D. & Carlson, S. (1986) J. Cell Biol. **102**, 23125–2136
11. Madara, J. L. (1987) Am. J. Physiol. **253**, C171–C175
12. Madara, J. L., Moore, R. & Carlson, S. (1987) Am. J. Physiol. **253**, C854–C861
13. Madara, J. L. & Pappenheimer, J. R. (1987) J. Membr. Biol. **100**, 149–164
14. Stevenson, B. R., Siliciano, J. D., Mooseker, M. S. & Goodenough, D. A. (1986) J. Cell Biol. **103**, 755–766
15. Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A. & Mooseker, M. S. (1988) J. Cell Biol. **106**, 1141–1149
16. Citi, S., Sabanay, H., Jakes, R., Geiger, B. & Kendrick-Jones, J. (1988) Nature (London) **333**, 272–276
17. Citi, S., Sabanay, H., Kendrick-Jones, J. & Geiger, B. (1989) J. Cell Sci. **93**, 107–122
18. Stevenson, B. R., Heintzelman, M. B., Anderson, J. M., Citi, S. & Mooseker, M. S. (1989) Am. J. Physiol. **257** (Cell Physiol. **26**), C621–C628
19. Stevenson, B. R., Anderson, J. M., Goodenough, D. A. & Mooseker, M. S. (1988) J. Cell Biol. **107**, 2401–2408
20. Curci, S. & Fromter, E. (1979) Nature (London) **278**, 355–357
21. Diamond, J. (1977) Physiologist **20**, 10–18
22. Fromter, E. & Diamond, J. M. (1972) Nature (London) **235**, 9–13
23. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
24. Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
25. Anderson, J. M., Van Itallie, C., Peterson, M., Stevenson, B. R., Goodenough, D. A. & Mooseker, M. S. (1989) J. Cell Biol., in the press
26. Johnson, D. A., Gautsch, J. W., Sportsman, J. R. & Elder, J. H. (1984) Gene Anal. Tech. **1**, 3–8
27. Duck-Chong, C. G. (1979) Lipids **14**, 492–497
28. Duffey, M. E., Hainau, B., Ho, S. & Bentzel, C. J. (1981) Nature (London) **294**, 451–453
29. Kuijpers, G. A. J., Van Nooy, I. G. P., Vossen, M. E. M., Stadhouders, A. M., Van Uyen, A., De Pont, J. J. H. H. M. & Bonting, S. L. (1985) Histochemistry **83**, 257–264
30. Mazariegos, M. R., Tice, L. W. & Hand, A. R. (1984) J. Cell Biol. **98**, 1865–1872
31. Ojakian, G. K. (1981) Cell (Cambridge, Mass.) **23**, 95–103

Received 22 May 1989/14 July 1989; accepted 27 July 1989