Tankyrase recruitment to the lateral membrane in polarized epithelial cells: regulation by cell–cell contact and protein poly(ADP-ribosylation)

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PARsylation [poly(ADP-ribosylation)] of proteins is implicated in the regulation of diverse physiological processes. Tankyrase is a molecular scaffold with this catalytic activity and has been proposed as a regulator of vesicular trafficking on the basis, in part, of its Golgi localization in non-polarized cells. Little is known about tankyrase localization in polarized epithelial cells. Using MDCK (Madin–Darby canine kidney) cells as a model, we found that E-cadherin-mediated intercellular adhesion recruits tankyrase from the cytoplasm to the lateral membrane (including the tight junction), where it stably associates with detergent-insoluble structures. This recruitment is mostly completed within 8 h of calcium-induced formation of cell–cell contact. Conversely, when intercellular adhesion is disrupted by calcium deprivation, tankyrase returns from the lateral membrane to the cytoplasm and becomes more soluble in detergents. The PARsylation activity of tankyrase promotes its dissociation from the lateral membrane as well as its ubiquitination and proteasome-mediated degradation, resulting in an apparent protein half-life of ~2 h. Inhi-

bition of tankyrase autoPARsylation using H₂O₂-induced NAD⁺ depletion or PJ34 [*N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylacetamide hydrochloride] treatment results in tankyrase stabilization and accumulation at the lateral membrane. By contrast, stabilization through proteasome inhibition results in tankyrase accumulation in the cytoplasm. These data suggest that cell–cell contact promotes tankyrase association with the lateral membrane, whereas PARsylation activity promotes translocation to the cytosol, which is followed by ubiquitination and proteasome-mediated degradation. Since the lateral membrane is a sorting station that ensures domain-specific delivery of basolateral membrane proteins, the regulated tankyrase recruitment to this site is consistent with a role in polarized protein targeting in epithelial cells.

Key words: calcium switch, epithelial membrane polarization, poly(ADP-ribose) polymerase (PARP), poly(ADP-ribosylation) (PARsylation), tankyrase, ubiquitination.

INTRODUCTION

Targeted delivery of newly synthesized membrane proteins involves sorting at sites that vary according to cell type [1]. In fibroblasts and other non-polarized cells, the Golgi complex is the major sorting site where cargo proteins are segregated into distinct vesicles for delivery to lysosomes, endosomes and the plasma membrane [2]. In epithelial cells where the cell surface is polarized into apical and basolateral domains, protein sorting also occurs on the plasma membrane and particularly at the junctional complex, the site of cell–cell adhesion. This region specifies the docking and fusion of post-Golgi vesicles carrying basolateral cargo proteins [3,4]. It also serves as a sorting station for a subset of apically destined proteins, which detour through the lateral membrane before being internalized and transcytosed to the apical domain [1,5–7]. Domain-specific delivery of membrane proteins in epithelial cells leads to the establishment of apico-basal membrane polarization, which is essential for vectorial transport of ions and solutes across epithelial monolayers [8].

Epithelial junctional complexes consist of AJs (adherens junctions) and TJs (tight junctions). The E-cadherin-based AJs are formed at the site of cell–cell contacts and provide the initial spatial landmark for membrane asymmetry [8]. To reinforce and maintain membrane polarization, AJs specify the localized assembly of a targeting patch that facilitates the docking/fusion of basolaterally directed transport vesicles [8]. AJs also provide the spatial cue that guides the assembly of TJs at the apex of the

lateral membrane, which block the mixing of protein and lipid constituents between apical and basolateral membranes [1,8]. Junctional complexes are highly enriched with membrane traffic regulators, including small GTPases of the Rab family, which modulate vesicle docking, and the Sec6–Sec8 complex (exocyst), which promotes vesicle fusion with the plasma membrane [1,8,9]. The recruitment of exocyst to junctional complexes is well characterized in polarized monolayers of MDCK (Madin–Darby canine kidney) cells [10]. Shortly upon contact formation between these cells, exocyst is effectively recruited from the cytoplasm to the entire length of the lateral membrane, a process that involves direct exocyst binding to E-cadherin [10,11]. As cell polarity develops and the nascent lateral surface expands, Sec6 and Sec8 become restricted to the apex of the junctional complexes, the site of continued basolateral membrane growth [10,11].

The dimerization of E-cadherin and hence the formation of junctional complexes between opposing cells are strictly calcium dependent [1,8]. Consequently, extracellular calcium depletion causes epithelial cells to lose membrane polarization and adopt a fibroblastic phenotype [12]. Concomitantly, various membrane traffic regulators such as rab3B, rab13 and the exocyst complex dissociate from the junctional complexes and relocate to the cellular interior (for references, see [8]). Despite the relocation, these proteins continue to regulate vesicular trafficking in the fibroblastic state [13]. For instance, in a fibroblastic strain of NRK (normal rat kidney) cells, components of the exocyst complex localize predominantly to the *trans*-Golgi network, where they

Abbreviations used: 3AB, 3-aminobenzamide; AJ, adherens junction; ANK, ankyrin repeat; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; IRAP, insulin-responsive aminopeptidase; MDCK, Madin–Darby canine kidney; NRK, normal rat kidney; PAR, polymers of ADP-ribose; PARP, poly(ADP-ribose); PARsylation, poly(ADP-ribosylation); PJ34, *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylacetamide hydrochloride; PNS, postnuclear supernatant; TJ, tight junction.

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regulate the production, instead of docking, of basolateral transport vesicles [4, 14].

Tankyrase is a molecular scaffold that localizes to the Golgi area in adipocytes and preadipocytes [15, 16] and to diffuse cytosolic vesicular structures in HeLa cells and a hepatoma cell line [17, 18]. Its modular structure consists of an ANK (an ankyrin repeat) domain, a dimerization module called SAM (sterile alpha motif) and a catalytic domain with PARP [poly(ADP-ribose) polymerase] activity [19]. The ANK domain contains five clusters of ANK repeats, each capable of binding to the sequence motif RXXPDG found in diverse tankyrase partners, including IRAP (insulin-responsive aminopeptidase) [20–24]. Tankyrase is proposed to regulate IRAP targeting in adipocytes on the basis of its IRAP binding and co-localization with IRAP storage vesicles in the Golgi region [15]. It has also been implicated in protein secretion in a hepatoma cell line, where tankyrase overexpression disrupts Golgi architecture and impairs the targeting of a glycoposphoinositol-linked protein marker [18]. Tankyrase is ubiquitously expressed [19], but its localization in polarized epithelial cells has not been examined.

The PARP domain of tankyrase catalyses PARsylation [poly(ADP-ribosyl)ation], a post-translational modification where the ADP-ribose moiety of NAD⁺ is transferred on to protein acceptors to form branching PAR (polymers of ADP-ribose) [19, 25]. The PARsylation activity of tankyrase is constitutive and is further enhanced by growth-factor signalling, owing to ERK (extra-cellular-signal-regulated kinase)-mediated phosphorylation of this PARP enzyme [15, 25]. Quantitatively the most important tankyrase substrate appears to be the enzyme itself [26]; however, the significance of this automodification is not yet clear.

Herein we show that tankyrase in MDCK cells is recruited from the cytoplasm to the lateral membrane upon formation of E-cadherin-based cell–cell contact. Conversely, the dissociation of tankyrase from the lateral membrane is stimulated by its PARsylation activity. PARsylation also promotes tankyrase turnover through the ubiquitin–proteasome system. Therefore tankyrase localization is regulated by both cell–cell contacts and cellular PAR metabolism.

MATERIALS AND METHODS

Cell cultures and treatments

Parental MDCK cells [obtained from the American Type Culture Collection (A.T.C.C.), Manassas, VA, U.S.A.] were cultured in DMEM (Dulbecco's modified Eagle's medium) (cellgro®; Mediatech Inc., Herndon, VA, U.S.A.) containing 0.45 % glucose, 0.37 % NaHCO₃, 2 mM L-glutamine (Gibco) and 5 % (v/v) FBS (fetal bovine serum; Omega Scientific, Inc., Tarzana, CA, U.S.A.). MDCK/T1 cells [26] were cultured in the above medium supplemented with hygromycin, G418 (both at 100 µg/ml) and doxycycline (100 ng/ml). –Ca medium consisted of calcium-free DMEM (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 5 µM CaCl₂ and 5 % FBS that had been extensively dialysed into 10 mM Tris/HCl, pH 7.5, and 120 mM NaCl. +Ca medium was the above supplemented with 1.8 mM CaCl₂. –Dox medium consisted of DMEM supplemented with 5 % tetracycline-free serum (Clontech, Palo Alto, CA, U.S.A.). +Dox medium was the above supplemented with doxycycline (100 ng/ml). Deoxycholate and 3-aminobenzamide were from Sigma (St. Louis, MO, U.S.A.).

Immunofluorescence analyses

Confluent MDCK/T1 monolayers were maintained in –Dox or +Dox medium for 1–2 days to induce or suppress tankyrase expression respectively. When indicated, the monolayers were

treated with PJ34 [*N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-*N,N*-dimethylacetamide hydrochloride; 80 µM; Inotek Pharmaceuticals Corp., Beverly, MA, U.S.A.], H₂O₂ (1.5 mM; Sigma) or MG-132 (10 µM; Sigma) in –Dox medium, or incubated in –Ca medium supplemented with CaCl₂ at the indicated concentration. For Triton-extraction studies, monolayers were overlaid for 10 min on ice with PBS or 1 % Triton X-100 in PBS, and soluble proteins were aspirated off. Cells were fixed in methanol for 5 min and acetone for 2 min (for Figures 5 and 8) or para-formaldehyde (for the other Figures), permeabilized with 0.2 % Triton X-100, and blocked as described in [26]. The primary antibodies were T1S (1 µg/ml [15]), GM-130 (1:40; Transduction Laboratories, Lexington, KY, U.S.A.) and ZO-1 (1:4; hybridoma supernatant; Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA, U.S.A.). FITC- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). The coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and wide-field images were acquired using a Carl Zeiss (Thornwood, NY, U.S.A.) Axioskop microscope. Alternatively, samples were mounted in Gelvatol [16 % (v/v) polyvinyl alcohol, 40 mM Tris/HCl, pH 8, and 60 % (v/v) glycerol] and images were acquired under a Zeiss LSM510 confocal microscope using an argon/krypton laser.

Iodixanol equilibrium gradient centrifugation

Parental MDCK cells grown in regular medium to confluence were switched to –Ca or +Ca medium for 38 h. PJ34 (80 µM) was added to +Ca medium as indicated 2 h prior to harvesting. Confluent MDCK/T1 cells maintained in –Dox medium were treated with 1.5 mM H₂O₂ where indicated. Cells were harvested at 4 °C by scraping in isotonic sucrose buffer as described in [10], homogenized using a 7-ml-capacity Dounce homogenizer (Wheaton Inc., Millville, NJ, U.S.A.) and clarified by centrifugation at 800 *g* for 10 min. Post-nuclear supernatants (3.5 mg of protein in 7 ml) were mixed with 7 ml of iodixanol [60 % (w/v); Accurate Chemicals, Westbury, NY, U.S.A.] and sealed in Ultra-Clear tubes [Beckman (Fullerton, CA, U.S.A.) no. 344322]. Self-generating gradients were formed by centrifugation at 300 000 *g* in an NVT65 rotor (Beckman) at 4 °C for 100 min.

Immunoblotting analyses

The primary antibodies included tankyrase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.; sc-8337 1:1000), ubiquitin (P4D1; Covance, Princeton, NJ, U.S.A.), poly(ADP-ribose) (1:1500; [26]), PARP1 (1:1000; [26]), Sec8 (Calbiochem, La Jolla, CA, U.S.A.; 14G1; 1:500), β-catenin (Santa Cruz; sc-7963; 1:10 000), E-cadherin (Sigma; clone DECMA-1; 1:800), GM-130 (BD Sciences; 1:500), IRAP (1:3000; [15]) and α-tubulin (Sigma, 1:1000). Secondary antibodies and enhanced chemiluminescence reagents were obtained as described in [26].

RESULTS

Tankyrase localizes to the lateral membrane in polarized epithelial cells

We investigated tankyrase localization in monolayers of MDCK cells, an established model for polarized epithelial cells [8]. To improve detection sensitivity, we immunostained the stable line MDCK/T1 expressing full-length tankyrase from a doxycycline-suppressible promoter [26]. When suppressed by doxycycline (+Dox), this line expressed tankyrase at the endogenous level of 1500 molecules/cell ([26] and results not shown). Doxycycline

Figure 1 Confocal immunofluorescence analysis of tankyrase (TNKS-1)

Confluent monolayers of MDCK/T1 cells were maintained in the presence (**A**) or absence (**B** and **C**) of doxycycline (Dox) for 2 days to suppress or induce recombinant tankyrase respectively and immunostained for tankyrase (green) and ZO-1 (red). (**A**) and (**B**) are confocal images taken at the plane of the TJ. (**C**) is an XZ image reconstructed from a Z-stack. The co-localization of tankyrase and ZO-1 is shown in yellow in the merged images. Arrowheads, TJs. Arrows, lateral aspect of the basolateral membrane.

withdrawal (–Dox) induced the expression by 40-fold, approaching the endogenous abundance in 3T3-L1 fibroblasts [26]. Using confocal microscopy to visualize MDCK/T1 monolayers stained with an anti-tankyrase antibody, we found only background staining in +Dox cells (Figure 1A). In contrast, –Dox cells showed a prominent linear staining at the cellular periphery that co-localized with the TJ protein ZO-1, as well as diffuse punctate staining in the cytoplasm (Figure 1B, left panel). Three-dimensional reconstruction of Z-axis sections showed that tankyrase covered the entire length of the lateral membrane (Figure 1C; arrows in the left panel) and was essentially absent from the apical and the basal aspects of the cell surface. Figure 1(C) also shows that tankyrase co-localized with ZO-1 (yellow pixels in the right panel), which resided at the apex of the lateral membrane (arrowheads in the middle panel). Therefore recombinant tankyrase localized to both the lateral membrane, including the TJ, and cytoplasmic vesicular structures in polarized MDCK cells.

Cell–cell contacts promote lateral recruitment of tankyrase

The lateral localization of tankyrase in polarized –Dox cells (Figure 1) was in sharp contrast with the predominantly Golgi

localization in fibroblastic cells [15,16], suggesting that the difference might be due to contact-induced membrane polarization in epithelial cells. To verify this, we depleted extracellular calcium to disrupt E-cadherin-mediated adhesion within –Dox monolayers and immunostained the resultant fibroblastic cells for tankyrase and the Golgi marker GM-130 [27]. In control cells maintained at physiological calcium concentrations (1.8 mM; Figure 2A), tankyrase staining on the lateral membrane was stronger than in the cytoplasm, where partial overlap with GM-130 was evident in occasional cells (arrows). When the calcium concentration was lowered to 100 μ M (Figure 2B), cytoplasmic tankyrase staining and the overlap with GM-130 became prominent in many cells (arrows). On further calcium deprivation (5 μ M; referred to as ‘–Ca’), cells retracted from their neighbours and rounded up as expected (Figure 2C), and tankyrase staining became predominantly cytoplasmic, again showing partial overlap with GM-130 (arrows). We therefore concluded that tankyrase recruitment to the lateral membrane depended on the formation of E-cadherin-mediated AJs, without which tankyrase defaulted to the cytoplasm and often resided in the vicinity of the Golgi complex, much like its physiological localization in fibroblasts [15,16]. This contact-dependent localization is reminiscent of the exocyst complex in the NRK cells, where the exocyst localizes to

Figure 2 Calcium-dependent tankyrase recruitment to the lateral membrane

Recombinant tankyrase was induced for 2 days in confluent MDCK/T1 monolayers maintained in $-$ Dox medium containing CaCl_2 at 1.8 mM (A), 100 μM (B) or 5 μM (C). Cells were immunostained for tankyrase (green; upper panels) and GM-130 (red; lower panels) and imaged under a wide-field fluorescence microscope. The merged images (middle panels) show overlapping green and red colours in yellow.

the *trans*-Golgi network in cells forming fibroblastic junctions and is recruited to the lateral membrane in those forming epithelioid junctions [14].

Lateral recruitment of tankyrase decreases solubility in detergents

In polarized epithelial cells, contact-induced recruitment of both exocyst and E-cadherin to the lateral membrane confers resistance to extraction by non-ionic detergents [10,28], presumably as a result of stable association with insoluble membrane microdomains (such as lipid rafts and caveolae) or incorporation into large complexes (such as the cytoskeleton and junctional complexes) [1,29–31]. To determine whether the lateral recruitment of tankyrase similarly decreased solubility, we extracted MDCK/T1 $-$ Dox monolayers with 1 % Triton X-100 prior to fixation and immunostaining. Whereas control cells displayed tankyrase staining in both the cytoplasm and the lateral membrane (Figure 3A, left panel), only the latter withstood Triton extraction (Figure 3A, right panel), indicating that the lateral recruitment of recombinant tankyrase resulted in association with detergent-insoluble structures.

Next we assessed whether endogenous tankyrase also became detergent-insoluble following recruitment to the lateral mem-

brane. To this end, we cultured parental MDCK cells in normal (+ Ca, 1.8 mM) or $-$ Ca medium to promote or decrease tankyrase association with the lateral membrane respectively. For each condition, monolayers were extracted with 1 % Triton X-100 to separate soluble (S) from insoluble proteins (P). Figure 3(B) shows that the integral membrane protein IRAP was entirely soluble, regardless of calcium concentrations, whereas E-cadherin and Sec8 were less soluble in +Ca monolayers, as previously reported [10,28]. Figure 3(B) also shows that tankyrase, too, was less soluble in +Ca samples. Calcium apparently affected the solubility of only lateral membrane proteins (tankyrase, E-cadherin and Sec8), since it did not affect the Golgi matrix protein GM-130 (Figure 3B). Collectively, our immunofluorescence and immunoblotting analyses supported the notion that tankyrase became less detergent-soluble once it had been recruited to the lateral membrane.

Cellular contacts promote stable tankyrase association with membranes

To confirm that contacts between epithelial cells induced a stable tankyrase association with the lateral membrane, we subjected $-$ Ca and +Ca cultures of parental MDCK cells to

Figure 3 Lateral-membrane localization decreases tankyrase (TNKS-1) solubility in detergents

(A) MDCK/T1 — Dox cells were overlaid with PBS (left panel) or PBS containing 1% Triton X-100 (right panel) on ice for 10 min. After removing solubilized proteins, residual proteins were fixed in 4% paraformaldehyde and immunostained for tankyrase. Wide-field images were acquired at the same setting for both samples. (B) Parental MDCK cells were cultured until 1–2 days post-confluence in regular medium, and switched to $-Ca$ or $+Ca$ medium overnight. Monolayers were overlaid with buffer A containing 1% Triton X-100 and 200 mM NaCl [15] ($300 \mu\text{l}/2 \text{ cm well}$) at 4°C for 12 min. Soluble fractions (S) were transferred into a tube containing $100 \mu\text{l}$ of $4 \times$ SDS/PAGE sample buffer. Insoluble residues (P) were detached in $400 \mu\text{l}$ of $1 \times$ SDS sample buffer and homogenized by passing through a 23-gauge needle. Equal volumes ($30 \mu\text{l}$) of S and P fractions were immunoblotted, and the insoluble/soluble (P/S) ratio was calculated on the basis of densitometric analysis. The bar graph to the right shows the mean P/S ratio ($\pm$ S.E.M., $n = 4$). P values were calculated using Student's two-tailed t test. A representative set of gels is shown to the left. E-cad, E-cadherin.

equilibrium-sedimentation analysis in self-forming iodixanol gradients. In these density gradients, cytosolic proteins sediment toward the bottom, whereas membrane-associated proteins, owing to the buoyancy of lipid bilayers, float toward the top [10,11]. Thus, in agreement with a previous report [10], we found that Sec8 recovery in the top two (membrane-associated) fractions was higher in $+Ca$ cells than in $-Ca$ cells, whereas the integral membrane protein E-cadherin was confined to the top two fractions in both samples (Figure 4A compared with Figure 4B). Figure 4 also shows that endogenous tankyrase in $-Ca$ cells sedimented toward the lower (cytosolic) portion of the gradients; only 16% was recovered in the top two fractions (Figure 4A). By contrast, as much as 35% of total tankyrase in $+Ca$ cells was recovered in the top two fractions — a level similar to that of Sec8 (Figure 4B). Therefore E-cadherin-mediated contacts promoted stable membrane association of tankyrase, much like the exocyst component Sec8.

To assess the kinetics of tankyrase–membrane association following the initiation of intercellular contacts, we added calcium to $-Ca$ monolayers to induce contacts and monitored the recovery of tankyrase in the top fraction of iodixanol gradients. We found

that calcium induced a rapid increase in membrane-associated tankyrase in parental MDCK cells (Figure 4C). Calcium also induced a gradual increase of total tankyrase abundance in the PNS (postnuclear supernatant) while having no effect on actin levels (Figure 4C). Calculation of the membrane/PNS ratio indicated that, within 2–8 h of calcium addition, tankyrase achieved more than 50% of its steady-state level of membrane association (Figure 4C). The same analysis of Sec8 showed a similar time course of calcium-induced membrane association (Figure 4C), in agreement with the half-life ($t_{1/2}$) of 3–6 h reported for this process [10]. We therefore concluded that tankyrase, like Sec8, underwent rapid cytosol-to-membrane redistribution following calcium-induced AJ formation and epithelial polarization.

PARsylation inhibition enhances tankyrase localization to the lateral membrane

Tankyrase can covalently modify itself through poly(ADP-ribosylation) [19,25], raising the possibility that autoPARsylation might regulate tankyrase recruitment to the lateral membrane. To

Figure 4 Calcium enhances the membrane association of endogenous tankyrase (TNKS-1) as assayed by iodixanol gradients

Parental MDCK cells grown to confluence in regular medium were switched to $-Ca$ (A and C) or $+Ca$ medium (B) for 38 h. In (C), $CaCl_2$ (1.8 mM) was added back and cells were harvested up to 8 h thereafter. For all samples, postnuclear homogenates (4 mg of protein) were resolved in 30% self-forming iodixanol gradients, and 1 ml fractions were collected from the top for immunoblotting (30 μ l/lane). In (A) and (B), the tankyrase and Sec8 contents of each fraction were quantified by densitometry and plotted as a percentage of the sum of all fractions. In (C), the first fraction [plasma membrane (PM), 60 μ l] and the input PNS (30 μ g of protein) were immunoblotted and quantified. The PM/PNS ratio at each time point was normalized to the ratio of a monolayer maintained in $+Ca$ medium for 60 h. The results shown are representative of three (A and B) or two (C) independent experiments.

abolish this automodification, we treated MDCK/T1 – Dox cells with increasing concentrations of PJ34, the most potent PARP inhibitor available commercially [32–34]. Figure 5(A) shows that 80 μ M PJ34 effectively abolished tankyrase autoPARsylation as assayed on anti-PAR immunoblots. (These blots typically reveal a smear of PARsylated tankyrase due to the heterogeneous extent of PARsylation.) Next we applied PJ34 (80 μ M for 2 h) to parental MDCK cells cultured in $+Ca$ medium, and assessed the impact on the membrane association of endogenous tankyrase using iodixanol gradients. Compared with untreated cultures fractionated in parallel (shown in Figure 4B), PJ34 treatment doubled tankyrase recovery in the top two fractions at the expense of the lower fractions (Figure 5B compared with Figure 4B). By contrast, the Sec8 distribution profile was largely unaffected (Figure 5B compared with Figure 4B), indicating that PJ34 selectively increased the membrane association of tankyrase. To ensure that this PJ34 effect was specifically due to inhibition of tankyrase autoPARsylation, we abrogated the autoPARsylation using a completely independent approach that depleted NAD^+ , the obligatory PARP co-substrate. We have shown that more than 85% of NAD^+ stores are depleted in MDCK/T1 – Dox cells challenged with H_2O_2 (1.5 mM for 2 h) [26], a genotoxin that potentially activates NAD^+ consumption by PARP1 (but not by tankyrase) [35]. As expected, H_2O_2 treatment inhibited tankyrase autoPARsylation (lane 10 in Figure 5C; [26]). Importantly, iodixanol-gradient analysis showed that H_2O_2 increased tankyrase recovery in the top two fractions (Figure 5C, lanes 6–7 compared with lanes 2–3), similar to the effect of PJ34 treatment. It appeared, therefore, that the increased membrane association of tankyrase

on H_2O_2 and PJ34 treatment was specifically due to the inhibition of tankyrase-mediated PARsylation.

Next we used detergent-insolubility as a read-out to assess the effect of PARP inhibition on the lateral localization of tankyrase. Figure 5(D) shows that PJ34 significantly increased the Triton-insolubility of endogenous tankyrase while having little effect on Sec8. A similar effect on tankyrase was also observed using a structurally distinct PARP inhibitor 3AB (3-aminobenzamide; 5 mM; results not shown), consistent with both PARP inhibitors enhancing detergent-resistant tankyrase association with the lateral membrane. As described below, both PARP inhibitors up-regulated tankyrase expression. To directly visualize the impact of PARP inhibition on tankyrase localization, we again turned to MDCK/T1 – Dox cells overexpressing the protein. Figure 5(E) shows that a 2 h treatment with either PJ34 (80 μ M; middle panel) or H_2O_2 (1.5 mM; lower panel) intensified tankyrase immunostaining at the lateral membrane, indicating that PARP inhibition promoted tankyrase accumulation at this locale. On the basis of these observations (Figures 5B–5E), we inferred that tankyrase dissociation from the lateral membrane was promoted by its PARP activity.

Tankyrase degradation is enhanced by its PARsylation activity

Tankyrase's PARsylation activity, besides affecting subcellular localization, apparently also destabilized the protein. First, in parental MDCK cells treated with PJ34 or 3AB, endogenous tankyrase was up-regulated within hours, whereas Sec8 was unaffected

Figure 5 PARP inhibition promotes tankyrase (TNKS-1) association with the lateral membrane

(A) MDCK/T1 monolayers maintained in $-$ Dox medium were treated with PJ34 at the indicated concentrations for 2 h. Whole-cell extracts (10% content of a 2 cm well/lane) were immunoblotted using anti-PAR (upper panel) and anti-tankyrase antibodies (lower panel). WB, Western blot. (B) Parental MDCK cells maintained in $+Ca$ medium in parallel with those used in Figure 4(B) were treated with PJ34 (80 μ M) for 2 h. Samples were fractionated in 30% iodixanol gradients and analysed in parallel with Figures 4(A) and 4(B). (C) MDCK/T1 monolayers maintained in $-$ Dox medium were treated with or without H_2O_2 (1.5 mM; 2 h). PNSs were fractionated in 30% iodixanol gradients as described in (B). The input material (PNS), the top two fractions (F1 and F2), and a mixture of the remaining fractions (F3-F12) were immunoblotted for tankyrase and Sec8 (30 μ l each; lanes 1-8). In lanes 9-10, whole-cell extracts were immunoblotted with anti-PAR and anti-tankyrase antibodies. (D) Monolayers of parental MDCK cells were treated with or without PJ34 (80 μ M) for 3 h. Triton-soluble (S) and -insoluble (P) pools of tankyrase and Sec8 were determined as in Figure 3(B). The bar graph shows the mean P/S ratio ($\pm$ S.E.M., $n = 4$). A representative set of gels is shown. (E) MDCK/T1 monolayers maintained in $-$ Dox medium were untreated (upper panel) or treated with PJ34 (80 μ M, middle panel) or H_2O_2 (1.5 mM, lower panel) for 2 h. Cells were fixed in methanol/acetone and immunostained for tankyrase. Wide-field immunofluorescence images were acquired at identical settings.

(Figure 6A, lanes 1-5). Moreover, in MDCK/T1 $-$ Dox cells treated with PJ34 (Figure 6A, lanes 6-7) or 3AB (results not shown), recombinant tankyrase expression was also up-regulated,

whereas PARP1 was unaffected (lanes 6-7). These effects on both endogenous and recombinant tankyrase suggest that PARP inhibitors stabilized the tankyrase protein, rather than enhancing

Figure 6 PARP inhibition and proteasome inhibition enhance tankyrase (TNKS-1) stability

(A) Parental MDCK cells (lanes 1–5) and MDCK/T1 – Dox cells (lanes 6–7) were treated with PJ34 (80 μ M; lanes 2, 3 and 7), 3-AB (5 mM, lane 5) or vehicle only (water for lanes 1 and 6; DMSO for lane 4) for the indicated durations. Cells were lysed in SDS/PAGE sample buffer (400 μ l/1 cm well) and samples were immunoblotted (40 μ l/lane) for the indicated proteins and for PAR epitopes. (B) Confluent MDCK/T1 monolayers were maintained in – Dox medium for 2 days to induce tankyrase expression. At time zero, cells were treated with doxycycline (100 ng/ml) either alone (lanes 2–4; thick line) or in combination with 80 μ M PJ34 (lanes 5–7; dotted line) or 10 μ M MG-132 (lanes 8–10; thin line). Whole-cell extracts were harvested at the indicated time points and immunoblotted for tankyrase. The abundance as determined by densitometry (mean $\pm$ S.E.M., $n=3$) was normalized to time zero and plotted in a semi-logarithmic scale at the top. The same gel was also immunoblotted for PAR epitopes, PARP1 and β -catenin. (C) MDCK/T1 – Dox cells grown in – Dox medium were pretreated with H₂O₂ (1.5 mM; lanes 3–4) or not (lanes 1–2) for 2 h. After rinsing off H₂O₂ using three washes with – Dox medium, cells were maintained in + Dox (lanes 2 and 4) or – Dox medium (lanes 1 and 3) for 3.5 h. Whole-cell extracts were immunoblotted for PARsylated species and the indicated proteins. Each lane represents 10% of the content from a 1 cm well.

gene transcription or translation. This is because the mRNAs encoding the two forms of tankyrase were driven by unrelated promoters and harboured distinct 5' and 3' untranslated sequences.

To examine the effect of PARP inhibition on tankyrase protein stability, we monitored the turnover of overexpressed tankyrase in MDCK/T1 – Dox cells after using doxycycline to switch off its mRNA synthesis. In control cells, doxycycline triggered a rapid decline in overall tankyrase levels (Figure 6B, lanes 1–4). On a semi-logarithmic scale, this decline exhibited an apparent $t_{1/2}$ of ~ 2 h (Figure 6B, upper panel, thick line). The actual $t_{1/2}$ of the tankyrase protein is likely to be even shorter, since doxycycline only inhibited the synthesis, but not the translation, of tankyrase mRNA. Interestingly, when combined with PJ34, doxycycline no longer down-regulated tankyrase abundance (Figure 6B, lanes 5–7). Instead, the abundance increased by 24% in cells treated with

both PJ34 and doxycycline for 10 h (dotted line). The tankyrase-stabilizing effect of PJ34 was specific, since it did not up-regulate β -catenin or PARP1 (Figure 6B, lanes 5–7). To confirm that PJ34 stabilized tankyrase by inhibiting its PARP activity, we again used H₂O₂-induced NAD⁺ depletion to inhibit tankyrase activity and assessed the impact on its turnover. Figure 6(C) shows that H₂O₂, as expected, enhanced PARP1 automodification and suppressed tankyrase automodification in MDCK/T1 – Dox cells (lane 1 compared with lane 3). The effect on tankyrase was less pronounced here than in Figure 5(C), because these cells were allowed to recover from H₂O₂ treatment for several hours. Importantly, H₂O₂ pretreatment protected tankyrase from doxycycline-induced down-regulation (lane 2 compared with lane 4). This H₂O₂ effect was not due to doxycycline inactivation by peroxide, since we extensively rinsed off H₂O₂ prior to adding

doxycycline. Moreover, we were able to recycle doxycycline in conditioned medium from H₂O₂-pretreated cultures (lane 4 of Figure 6C), apply it to drug-naïve MDCK/T1 – Dox cells, and demonstrate suppression of tankyrase expression (results not shown). Collectively our data indicate that both H₂O₂ and PJ34 stabilized tankyrase by inhibiting its PARP activity.

To explore the machinery that rapidly turned over tankyrase, we treated MDCK/T1 – Dox cells concurrently with doxycycline and the proteasome inhibitor MG-132 (carbobenzoxy-L-leucyl-L-leucyl-leucinal). Figure 6(B) shows that MG-132 completely reversed doxycycline-mediated tankyrase down-regulation (lanes 8–10, upper panel, thin continuous line) and actually caused a 56 % up-regulation over 10 h. Unlike PARP inhibition, MG-132 did not inhibit tankyrase autoPARsylation, but did up-regulate β -catenin (lanes 8–10), an established proteasome substrate [36]. Therefore, although proteasome inhibition and PARP inhibition both stabilized the tankyrase protein, they apparently blocked distinct steps in the degradation pathway.

As a complementary strategy to inhibit tankyrase PARsylation, we mutated its catalytic center using an M1207V substitution. Like the equivalent tankyrase-2 mutation [25], this substitution completely inactivated tankyrase according to *in vitro* PARP assays (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/399/bj3990415add.htm>, lane 2 compared with lane 4). However, when inducibly expressed from a tet-off promoter in MDCK cells, PARsylation of this M1207V mutant was evident (Supplementary Figure S3, lane 2, at <http://www.BiochemJ.org/bj/399/bj3990415add.htm>), albeit less extensive than wild-type control (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/399/bj3990415add.htm>), indicating that the mutant was modified *in-trans* by endogenous tankyrase and/or tankyrase-2. In support of this intermolecular PARsylation, both tankyrase and tankyrase-2 are known to form homo- and hetero-oligomers [18,25], and analogous *trans*-autoPARsylation has been established for PARP1 [37]. As expected, PJ34 treatment inhibited the PARsylation of this mutant (Supplementary Figure S3, lane 3) and also up-regulated its abundance (results not shown). Consequently, we were unable to use this M1207V mutant to investigate the role of PARsylation in tankyrase turnover.

Tankyrase PARsylation promotes ubiquitination

Unstable proteins in mammalian cells are turned over predominantly by the ubiquitin–proteasome pathway [38]. The short $t_{1/2}$ of tankyrase and its stabilization on proteasome inhibition indicated that tankyrase underwent ubiquitination, leading to degradation by the proteasome. To demonstrate this, we affinity-precipitated tankyrase from MDCK/T1 – Dox cells by incubating extracts with immobilized GST-IRAP_{78–108} [glutathione S-transferase-IRAP-(78–108)-peptide], a fusion protein that binds tankyrase [15]. Anti-ubiquitin immunoblotting of the affinity precipitates revealed a smear of ubiquitinated tankyrase that migrated near the 250 kDa marker (Figure 7, upper panel, lane 1), indicative of conjugation of the 160 kDa tankyrase with multiple units of the 8 kDa ubiquitin. As expected, this smear of ubiquitinated tankyrase was not detected when tankyrase expression in MDCK/T1 cells was suppressed using doxycycline or when extracts were incubated with GST instead of GST-IRAP (results not shown).

Because tankyrase turnover is mediated by the ubiquitin–proteasome system and is suppressed upon PARP inhibition, a likely scenario is that PARP inhibitors abrogated tankyrase ubiquitination. Indeed, Figure 7 shows that PJ34 treatment not only abolished tankyrase autoPARsylation (lane 2, lower panel), but also decreased its ubiquitination by 93 % (upper panel, average for three observations), indicating that autoPARsylation promoted

Figure 7 Ubiquitination of tankyrase (TNKS-1) is decreased by PJ34

Confluent monolayers of MDCK/T1 were maintained in –Dox medium for 2 days to induce tankyrase expression. After treatment with PJ34 (80 μ M; 2 h; lane 2) or without (lane 1), cells were lysed in buffer A (1 ml/15cm plate) supplemented with 1 % deoxycholate. Extracts were precleared by incubating with GST protein (50 μ g/ml extracts) for 30 min, and centrifuged at 13 000 *g* for 10 min. Soluble extracts were incubated with GST-IRAP_{78–108} resins [25] (15 μ g of protein/ml extracts) to precipitate tankyrase. The precipitates were immunoblotted using anti-ubiquitin (Ub) (upper panel) and anti-tankyrase (middle panel) antibodies. Extracts were immunoblotted using anti-PAR antibodies (lower panel). WB, Western blot.

tankyrase ubiquitination. In the experiment described in Figure 7, we lysed cells in the presence of the ionic detergent deoxycholate (1 %) to overcome Triton-insolubility of tankyrase in PJ34-treated cells (shown in Figure 5D).

Cytosolic translocation of tankyrase precedes degradation

Given that tankyrase-mediated PARsylation promoted both its cytosolic translocation from the lateral membrane (Figure 5) and its degradation by the ubiquitin–proteasome system (Figures 6 and 7), we hypothesized that cytosolic translocation preceded proteolysis. We therefore predicted that, upon blocking tankyrase turnover with proteasome inhibitors, tankyrase should accumulate in the cytoplasm rather than on the lateral membrane. Indeed, immunofluorescence analysis of MDCK/T1 – Dox cells showed that MG-132 treatment intensified cytoplasmic tankyrase staining in a diffuse vesicular pattern (Figure 8A compared with Figure 8B, left-hand panels). Concomitantly, the staining became relatively indistinct on the lateral membrane, even though the integrity of the lateral membrane itself remained intact, as evidenced by ZO-1 staining (Figure 8B, middle panel).

DISCUSSION

The present study shows that E-cadherin-based contacts between MDCK cells signal the recruitment of tankyrase to detergent-insoluble compartments associated with the lateral membrane, and that disruption of these contacts causes tankyrase to return to the cytoplasm. Our data also indicate that the PARP activity of tankyrase promotes its dissociation from the lateral membrane, which is followed by ubiquitination and proteasome-mediated degradation.

The immunostaining of overexpressed tankyrase on the lateral membrane is likely indicative of endogenous protein, because the extent of overexpression was modest (40-fold) in our MDCK/T1 – Dox cells and did not exceed the endogenous abundance in 3T3-L1 fibroblasts [26]. More importantly, the two conditions that enhanced tankyrase immunostaining on the lateral membrane (i.e. cell–cell contact and PARP inhibition; Figures 2 and 5E) also increased the membrane association of endogenous tankyrase

Figure 8 Cytoplasmic immunostaining of tankyrase (TNKS-1) is enhanced by MG-132

Monolayers of MDCK/T1 cells maintained in —Dox medium for 2 days to induce tankyrase were untreated (**A**) or treated with 10 μ M MG-132 (**B**) for 90 min. Cells were co-stained for tankyrase (green) and ZO-1 (red). Wide-field immunofluorescence images were acquired at the same setting for both samples.

as revealed in iodoxanol gradients (Figures 4B and 5B). Lastly, both immunostaining of recombinant tankyrase (Figure 3A) and fractionation of endogenous tankyrase (Figure 3B) indicate that lateral membrane-associated tankyrase is relatively resistant to detergent extraction. Therefore we conclude that tankyrase is recruited to the lateral membrane in polarized epithelial cells. This localization pattern was not observed in adipocytes or HeLa cells [15,17], presumably because of the absence of E-cadherin expression and hence the lack of polarization [39,40].

Tankyrase on the lateral membrane appears to exchange rapidly with the cytosolic pool. Thus, within hours after calcium-deprived MDCK cells re-establish contacts, cytoplasmic tankyrase undergoes translocation to the lateral membrane (Figure 4C). Furthermore, the short $t_{1/2}$ of tankyrase (~ 2 h; Figure 6B) requires that nascent protein be rapidly recruited to the lateral membrane to offset turnover from that site. Lastly, tankyrase showed increased lateral-membrane localization within 2 h of PJ34 or H_2O_2 treatment (Figures 5B, 5C and 5E), indicating that PARP inhibition blocks the otherwise rapid dissociation of tankyrase from the lateral membrane. Consistent with this notion of rapid tankyrase shuttling to and from the lateral membrane, MDCK cells endocytose and replenish half of their cell surface each hour [41].

How PARsylation modulates the lateral localization of tankyrase remains to be further investigated. AutoPARsylation, the predominant tankyrase-mediated reaction, could conceivably increase its affinity for cytosolic proteins or, conversely, preclude its association with components of lateral membranes. In either scenario, PARP inhibition would induce tankyrase accumulation on the lateral membrane, as observed (Figure 5). Alternatively, tankyrase could PARsylate other proteins that in turn impact tankyrase localization.

Besides promoting cytoplasmic translocation, tankyrase's PARP activity also promotes its ubiquitination and degradation

(Figures 6 and 7). To link these PAR-dependent events mechanistically, we propose that PARsylation of tankyrase promotes its lateral membrane-to-cytosol translocation, which in turn leads to ubiquitination and proteasome-mediated degradation. This sequence of events (PARsylation $\rightarrow$ cytoplasmic translocation $\rightarrow$ ubiquitination $\rightarrow$ degradation) can explain why PARP inhibition and proteasome inhibition both stabilized tankyrase, but each resulted in accumulation at a distinct site (lateral membrane compared with cytoplasm respectively) (Figures 5E and 8). In the proposed sequence, tankyrase PARsylation is coupled to ubiquitination not directly, but rather through promoting cytosolic translocation. Therefore PARP inhibitors are not expected to totally abolish ubiquitination, since they do not completely eliminate tankyrase from the cytosol (Figures 5B and 5E). The notion that non-PARsylated tankyrase can undergo ubiquitination is supported by the presence of the destabilizing motif PEST in tankyrase amino acids 79–184 according to sequence analysis by www.at.emblnet.org/emblnet/tools/bio/PESTfind. Consisting of multiple proline (P), glutamic acid (E), serine (S) and threonine (T) residues flanked by basic residues, this motif often serves to tag proteins for degradation by the ubiquitin–proteasome system [42]. This destabilizing role of the PEST motif is not known to depend on PARsylation, further supporting the notion that PARsylation destabilizes tankyrase indirectly by causing cytoplasmic translocation. We propose that tankyrase destabilization by the ubiquitin–proteasome system provides a mechanism for rapidly varying tankyrase levels in response to physiological needs. Thus the stimulation of tankyrase ubiquitination/degradation by autoPARsylation allows a regulation of its abundance by cellular levels of NAD^+ (the PARP co-substrate) and nicotinamide (an endogenous PARP inhibitor).

Tankyrase is thought to regulate vesicular trafficking in non-polarized cells [15,18], but its functions in polarized epithelial

cells remain to be identified. Having used the MDCK model to show that tankyrase resembles exocyst in terms of the kinetics of contact-induced lateral recruitment and the resulting resistance to detergent extraction, we propose that tankyrase, like exocyst, facilitates the polarized delivery of membrane proteins to the basolateral domain. Moreover, the effects of PARsylation on tankyrase localization and stability suggest that the proposed protein-trafficking function is likely modulated by NAD⁺ and nicotinamide metabolism.

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