Connexin43 phosphorylation at S368 is acute during S and G_2/M and in response to protein kinase C activation

Joell L. Solan[1], Matthew D. Fry[2], Erica M. TenBroek[3] and Paul D. Lampe[1,*]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA, and Department of Pathobiology, University of Washington, WA 98195, USA
[2]Cell Signaling Technology, Beverly, MA 01915, USA
[3]Genetics, Cell Biology and Development, University of Minnesota, St Paul, MN 55108, USA
*Author for correspondence (e-mail: plampe@fhcrc.org)

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Summary

Phorbol esters such as 12-*O*-tetradeconylphorbol-13-acetate (TPA) activate protein kinase C, increase Connexin43 (Cx43) phosphorylation, and decrease cell-cell communication via gap junctions in many cell types. Previous work has implicated protein kinase C (PKC) in the direct phosphorylation of Cx43 at S368, which results in a change in single channel behavior that contributes to a decrease in intercellular communication. We have examined Cx43 phosphorylation in several cell lines with an antibody specific for phosphorylated S368. We show that this antibody detects Cx43 only when it is phosphorylated at S368 and, consistent with previous results, TPA treatment causes a dramatic increase in phosphorylation at S368. However, in some cell types, the increased phosphorylation at S368 did not cause a detectable shift in migration as compared with the nonphosphorylated Cx43. Immunofluorescence showed increased S368 immunolabeling in cytoplasmic and plasma membrane structures in response to TPA. Immunoblot analysis of synchronized cells showed increased phosphorylation at S368 during S and G_2/M phases of the cell cycle. S-phase cells contained more total Cx43 but assembled fewer functional gap junctional channels than G_0-phase cells. Since M-phase cells also communicate poorly and contain few assembled gap junctions, phosphorylation at S368 appears to be negatively correlated with gap junction assembly. Thus, both gap junctional communication and S368 phosphorylation change during S phase and G_2/M, implying that phosphorylation at S368 might play a role in key cell-cycle events.

Key words: Gap junctions, Connexins, Tumor promoter, Phosphorylation, Carcinogenesis

Introduction

Gap junctions are specialized membrane domains composed of collections of channels that directly connect neighboring cells (Willecke et al., 2002). These pathways provide for the cell-to-cell diffusion of small molecules, including ions, amino acids, nucleotides and second messengers (e.g. Ca^{2+}, cAMP, cGMP, IP_3). Recent studies on the targeted disruption of connexin genes, which encode vertebrate gap junction channel proteins, provide strong support for roles in cell growth control and embryonic development, as well as the transmission of metabolites and electrical signals between cells (Willecke et al., 2002).

Transient changes in gap junctional communication, probably regulated by signaling cascades, have been observed and appear necessary for normal cell cycling. For example, gap junctional communication was reported to be moderate during G_1/S, increased through S and decreased in G_2/M (Bittman and LoTurco, 1999; Stein et al., 1992). The downregulation of junctional communication during G_2/M has been correlated with increased $p34^{cdc2}$ kinase-dependent phosphorylation of Cx43 (Kanemitsu et al., 1998; Lampe et al., 1998a) and redistribution of Cx43 from gap junctions to the cytoplasm (Lampe et al., 1998a; Xie et al., 1997). Gap junctional structures reassemble and communication is gradually restored as cells proceed through G_1 (Stein et al., 1992; Xie et al., 1997). Cx43 is phosphorylated at multiple serine residues in vivo (Berthoud et al., 1992; Brissette et al., 1991; Crow et al., 1990; Kadle et al., 1991; Laird et al., 1991; Musil et al., 1990), and upon phosphorylation, Cx43 migration in polyacrylamide gel electrophoresis (SDS-PAGE) is reduced. Although apparently not required for the formation of functional channels (Dunham et al., 1992; Fishman et al., 1991), phosphorylation of gap junction proteins appears to regulate channel function (gating) and the rates of channel assembly and turnover (Brissette et al., 1991; Kwak et al., 1995a; Kwak et al., 1995b; Kwak et al., 1995c; Lampe, 1994; Lampe et al., 2000).

In the sustained absence of connexin expression, tumorigenesis is enhanced (Laird et al., 1999; Moennikes et al., 1999). The correlation between neoplastic transformation and reduced gap junctional communication (Atkinson et al., 1981; Azarnia and Loewenstein, 1984; de Feijter et al., 1990) has led to the hypothesis that reduced cell-cell communication is a critical step in multistage carcinogenesis (Fitzgerald and Yamasaki, 1990; Trosko et al., 1990). PKC has received considerable attention because PKC activators (e.g. TPA), which promote tumorigenesis, both increase Cx43

phosphorylation and decrease gap junction communication in several different cell types (Berthoud et al., 1992; Berthoud et al., 1993; Brissette et al., 1991; Lampe, 1994; Reynhout et al., 1992). PKC has been shown to phosphorylate Cx43 at S368, and this site has been shown to underlie a TPA-induced reduction in intercellular communication and alteration of single channel behavior (Lampe et al., 2000). However, in some cell types, TPA treatment did not lead to a shift in Cx43 mobility in SDS-PAGE (which is thought to indicate increased Cx43 phosphorylation) but did change gap junctional communication (e.g. Rivedal and Opsahl, 2001), leading to confusion as to the role of Cx43 phosphorylation in this process.

Here, we report that Cx43 phosphorylation at S368 was indeed increased by TPA treatment in all cell types tested, but that Cx43 mobility was not significantly affected in some. Furthermore, S368 phosphorylation was increased during key stages of the cell cycle where gap junctional assembly is reduced. Thus, in addition to its role in the regulation of gap junction channel gating, phosphorylation at S368 was negatively correlated with gap junction assembly.

Materials and Methods

Cell line maintenance and transfection

Normal rat kidney (NRK) epithelial cells (NRK-E51, American Type Culture Collection, ATCC, Rockville, MD), Chinese Hamster Ovary (CHO) and HeLa cells were cultured in DMEM (Mediatech, Pittsburgh, PA) supplemented with 5% fetal calf serum and antibiotics in a humidified 5% CO_2 environment. A serine-to-alanine site 368 mutant Cx43 cDNA was generated using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene, La Jolla, CA) and was then subcloned into the bicistronic expression vector pIREShyg (Clontech Laboratoroes, Palo Alto, CA) and transfected into the HeLa cell lines. Stably transfected clones were isolated by repeated dilution subcloning in the presence of the selective antibiotic hygromycin (200 μg/ml).

Metabolic labeling and Cx43 immunoprecipitation

NRK cells were cultured, metabolically labeled with [^{32}P]orthophosphate (ICN, 64014L) or ^{35}S-Trans label (ICN, 5100607), and immunoprecipitated essentially as previously described (TenBroek et al., 2001). Briefly, cells were labeled with [^{32}P]orthophosphate at 1.0 mCi/ml for 3 hours in phosphate-deficient medium (Gibco-Invitrogen, Grand Island, NY) and, where indicated, were treated with 50 ng/ml TPA during the final 30 minutes. Alternatively, cells were washed three times and labeled with ^{35}S-Trans label at 0.1 mCi/ml for 3 hours in methionine-free media (Gibco-Invitrogen) and, where indicated, were treated with 50 ng/ml TPA during the final 30 minutes. The cells were rinsed in PBS, lysed in RIPA buffer [25 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 50 mM NaF, 500 μM Na_3VO_4, 0.25% Triton X-100, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 1× Roche Complete protease inhibitors], clarified with protein A beads, and immunoprecipitated with p368 antibody [a rabbit anti-Phospho-Cx43 (Ser368) antibody #3511; Cell Signaling Technology, Beverly, MA], rabbit antibody C6219 from Sigma (St Louis, MO) and/or monoclonal Cx43CT1 antibody. Cx43CT1 antibody is an antibody prepared to a peptide representing the last 23 amino acids of Cx43 (described in Cooper and Lampe, 2002). Cx43CT1 behaves like antibody 13-8300 from Zymed, which was prepared to the same region of Cx43, in that it immunoprecipitates primarily the 'NP' form of Connexin unless cells are treated with TPA, when slower migrating forms were detected (Cruciani and Mikalsen, 1999). After four washes in RIPA buffer, the immunoprecipitates were treated with Laemmli sample buffer and run via SDS-PAGE (10% polyacrylamide, Tris-glycine gels).

Immunoblotting

Cells were lysed in sample buffer containing 50 mM NaF, 500 μM Na_3VO_4, 2 mM PMSF and 1× Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN) and cellular proteins were separated by SDS-PAGE on 10% Tris-glycine gels. For alkaline phosphatase treatment, cells were lysed in 0.2% SDS, 2 mM PMSF and 1× protease inhibitors, and briefly sonicated followed by addition of one-tenth volume of 10× phosphatase buffer (M183A; Promega, Madison, WI) and incubation with 10 units of calf intestinal alkaline phosphatase (M182A; Promega) for 1 hour at 37°C. After electrophoresis, protein was transferred to nitrocellulose, the membrane was blocked, and antibodies were incubated as previously indicated (Lampe et al., 1998a). Primary and secondary antibodies utilized were p368 antibody, mouse anti-Cx43 (Cx43NT1 described in Goldberg et al., 2002), mouse anti-vinculin (Sigma), peroxidase-conjugated donkey anti-mouse or mouse anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Where indicated, the blots were 'stripped' for 30 minutes at 50°C in 62.5 mM Tris pH 6.8, 1% SDS and 5% β-mercaptoethanol buffer followed by washing for 2 hours with at least six changes of PBS. Signal was visualized with SuperSignal West Pico or Femto Chemiluminescent Substrate (Pierce Chemicals, Rockford, IL) followed by exposure to Kodak Biomax MR film. Densitometry of autoradiographs was performed on a Macintosh G3 using a Sharp JX-325 scanner to collect the image and the public domain NIH Image program (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image).

Immunofluorescence

NRK cells were untreated or treated with TPA for 30 minutes at 37°C, washed twice in PBS, and fixed in cold methanol/acetone (50:50) for 1 minute followed by blocking for 1 hour in 1% bovine serum albumin in PBS. Cells were incubated with anti-Cx43 antibody p368 and/or Cx43IF1 (see Cooper and Lampe, 2002; TenBroek et al., 2001) in blocking solution for 1 hour. Following several PBS washes, the cultures were incubated with Alexa594-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) and/or fluorescein isothiocyanate-conjugated donkey anti-mouse antibody (Jackson Immunoresearch Laboratories) for 30-60 minutes and counterstained with DAPI (Molecular Probes), followed by several washes in PBS. The coverslips were mounted onto slides with DABCO antifade medium [25 mg/ml of 1,4-diazobicyclo-(2,2,2)octane (Sigma) diluted in Spectroglycerol (Kodak) and 10% PBS, pH 8.6] and viewed with a Nikon Diaphot TE300 fluorescence microscope, equipped with a 40× (1.3 n.a.) oil objective and a Princeton Instruments cooled digital camera driven by an attached PC and Metamorph imaging software.

Cell synchronization

G_0 cells were prepared by contact-inhibiting NRK cells at least 3 days past confluency without addition of fresh media. To obtain G_1 cells, confluent cells were trypsinized, then diluted to 60-80% confluency and allowed to progress 8-10 hours for early G_1 and 14-16 hours for late G_1. Cell-cycle analysis showed that by 18 hours these cells begin to enter S phase. For preparation of G_1/S, S, G_2 and G_2/M cells, confluent cells were trypsinized then diluted to 60-80% confluency in media containing 1 mM thymidine for 16 hours to induce a G_1/S block. Cells were released from G_1/S by washing and replacement of 37°C complete media. Cell-cycle analysis showed that S phase lasts 4-6 hours in these cells and that cells cycle through G_2/M to G_1 by 9-11 hours after washout. We typically observed 70-90% synchrony as

cells progress through S to G_1. Cell-cycle analysis was performed by fluorescence activated cell sorting. Specifically, cells were trypsinized, then pelleted in PBS with 2% fetal bovine serum and fixed in 70% EtOH. Cells were pelleted, washed and incubated with 5 μg/ml RNase at 37°C for 30 minutes, and then stained with 50 μg/ml propidium iodide on ice for 1 hour. DNA content was assessed on a Becton Dickinson FACScalibur and data analyzed using CellQuest software.

Gap junctional communication/assembly

Gap junctional communication was assayed via dye transfer according to published methods using either an assembly-preloading assay with calcein-AM (Lampe et al., 1998b) or by microinjection of fluorescent dyes. Briefly, for the preloading assay, one 10 cm plate of NRK cells was labeled with 0.5 μM calcein-AM (Molecular Probes), the cell-permeant ester of calcein that is cleaved to membrane-impermeant calcein by cellular esterases. Three other culture plates were labeled with 0.25 μM DiI (Molecular Probes). After washing twice with PBS, the two populations of cells were each trypsin/EDTA suspended, treated with trypsin inhibitor and pelleted. The cells were suspended in the appropriate media, mixed, plated on culture dishes and placed in a 37°C incubator. Cells were allowed to adhere for 2 hours then digital images of calcein and DiI were captured. The assignment of a cell as an acceptor of dye via transfer rather than a poorly loaded or leaking donor is checked by digitally overlaying images of DiI and calcein fluorescence. If a cell adjacent to a calcein-loaded, DiI-negative cell contains both punctate DiI and more-diffuse calcein fluorescence, gap junction assembly and dye transfer occurred. If a DiI-labeled cell adjacent to a calcein-loaded cell does not contain calcein, then dye transfer did not occur at that interface. A more-complete description of this assay is published elsewhere (Lampe, 1994; Lampe et al., 1998b). The fraction of cells that transferred dye were determined by dividing the number of DiI-labeled cells that contained calcein (i.e. transfers) by the number of cell interfaces between calcein-loaded and DiI-labeled cells (i.e. total).

Dye transfer in established cultures was analyzed by microinjection of a 10 mM solution of each of the gap junction permeable dyes, Alexa hydrazide 488 and 594 (M_r=570.5 and 758.8, respectively; Molecular Probes) in 0.2 M KCl. The dyes were microinjected using a 5 millisecond pulse of air at 10 psi from a General Valve Picospritzer II, and the number of cells receiving dye was analyzed after 10 minutes using the imaging system described above.

Results

Phospho-Cx43-Ser368 (p368) antibody is specific for phosphorylation of S368

We have developed an antibody that reacts with Cx43 when it is phosphorylated at S368. To test the specificity of the p368 antibody, HeLa cells that did not express any Cx43 were stably transfected with wild-type (wt) Cx43 or Cx43 containing a serine-to-alanine substitution at position 368 (S368A) and were examined by immunoblot analysis. Previously, we have shown that cells treated with TPA showed increased phosphorylation on Cx43, especially at S368 (Lampe et al., 2000). HeLa cells, either treated with TPA for 30 minutes or untreated, were washed and directly lysed in sample buffer, and whole-cell lysates were immunoblotted and probed for Cx43 content using the p368 antibody (rabbit) followed by stripping/reprobing of the blot using the mouse monoclonal Cx43 antibody Cx43NT1 (Fig. 1). The anti-Cx43 probing of HeLa cells expressing wt Cx43 or S368A Cx43 (Fig. 1, α-Cx43, CON lanes) showed typical migration patterns of Cx43 in SDS-PAGE, with multiple bands representing different

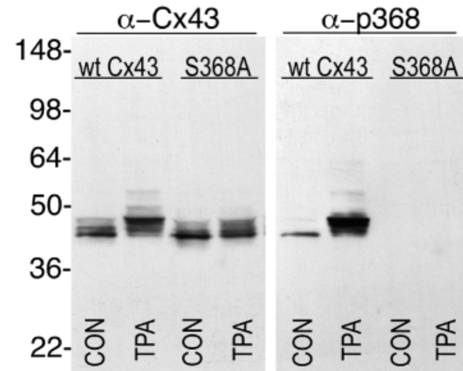

Fig. 1. The p368 antibody reacts with Cx43 only when S368 is present. Shown is an immunoblot of whole cell lysates from HeLa cells transfected with wild-type (wt) Cx43 or Cx43 containing a S368A mutation. Cells were either incubated in the presence (TPA) or absence (CON) of 50 ng/ml TPA for 30 minutes. The immunoblot was probed with either an antibody to the N-terminal region of Cx43 (α-Cx43) or the anti-p368 antibody (α-p368). Positions of the molecular weight markers are shown on the left.

phosphorylation states of Cx43. The predominant nonphosphorylated (NP) form migrates fastest, followed by slower-migrating phosphorylated forms, often referred to as P1, P2, etc., which can be converted to the faster-migrating form via alkaline phosphatase treatment (Berthoud et al., 1992; Brissette et al., 1991; Kadle et al., 1991; Laird et al., 1991; Lampe, 1994; Musil et al., 1990). Upon TPA treatment, essentially all of the Cx43 migration in HeLa cells containing wt Cx43 was shifted to slower-migrating species as has been observed previously in many different cell types (reviewed by Lampe and Lau, 2000). HeLa cells containing Cx43 with a S368A site-directed mutation responded much less extensively to TPA treatment (Fig. 1, α-Cx43), indicating that modification/phosphorylation on S368 affects the mobility shift, at least in these HeLa cells. Probing this same blot with the p368 antibody (Fig. 1, α-p368) showed that cells containing wt Cx43 had a low level of Cx43 phosphorylated at S368 (CON) that appeared to migrate similarly to NP Cx43. Upon TPA treatment, both a tenfold increased signal and a shift in migration of wt Cx43 was observed with the p368 antibody. Lysates from cells containing Cx43 with the S368A mutation showed no p368 antibody reactivity regardless of TPA treatment or long exposure of blots.

To verify further that this antibody recognized a phosphorylated species of Cx43, NRK cell lysates from control cells and TPA-treated cells were incubated with alkaline phosphatase and analyzed by immunoblot. As above, immunoblots were first processed with the p368 antibody, then stripped and reprobed with the anti-Cx43 antibody, allowing precise alignment and determination of the extent of migration of the bands. Immunoblots incubated with anti-Cx43 showed little change in response to TPA treatment (Fig. 2, α-Cx43 panel; compare CON and TPA lanes). When these cell lysates were incubated with alkaline phosphatase, all of the Cx43 migrated as the NP form (Fig. 2, α-Cx43 panel, AP lanes), which is consistent both with effective alkaline phosphatase treatment and with what has been shown by other investigators, as noted above. Processing of the blot with anti-p368 antibody

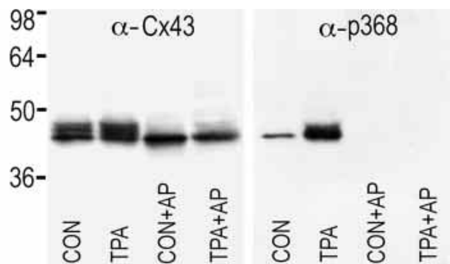

Fig. 2. The p368 antibody reacts with Cx43 only when it is phosphorylated at S368. Untreated (CON) or TPA-treated cells were lysed in sample buffer or treated with alkaline phosphatase (+AP lanes) prior to SDS-PAGE and immunoblotting. The blot was probed with the p368 antibody (α-p368 panel) followed by stripping and reprobing with the Cx43 antibody (α-Cx43 panel).

showed a sixfold increase in signal upon TPA treatment (Fig. 2, α-p368 panel; compare CON and TPA) and this signal was completely lost upon alkaline phosphatase treatment (Fig. 2, α-p368 panel, AP lanes). These data show that the p368 antibody reactivity appears to be specific for S368 only when it is phosphorylated (i.e. it is phosphorylation-state specific) and that a dramatic increase in phosphorylation at S368 is generated in response to TPA.

The 'NP' form of Cx43 can be phosphorylated on S368

Figs 1 and 2 indicate that an isoform of Cx43 that migrated similarly to NP Cx43 reacted with the p368 antibody. To determine more directly whether a phosphorylated species migrated to the same extent as the NP form of Cx43, we performed metabolic labeling on NRK cells with [^{32}P]orthophosphate or [^{35}S]methionine. Cx43 from [^{32}P]orthophosphate-labeled cells was immunoprecipitated, run on SDS-PAGE and blotted to nitrocellulose. These samples were analyzed first by autoradiography (Fig. 3, ^{32}P panel) and then immunoblot analysis using p368 (α-p368 panel) and Cx43NT1 (α-Cx43) monoclonal antibodies. In the autoradiograph, Cx43 immunoprecipitated from untreated cells showed two band, indicated as P1 and P2, whereas cells treated with TPA showed a more-broad phosphorylation pattern some of which appeared to migrate at the same position as the NP form. The α-p368 panel, which represents the chemiluminescent signal obtained from the same blot probed with p368 antibody, shows a dramatic TPA-dependent increase in signal co-migrating with the NP form, whereas probing the same blot with the α-Cx43 antibody showed minor differences in the typical pattern for Cx43 with or without TPA treatment. Thus, the TPA-dependent increase in Cx43 phosphorylation levels found by autoradiography was not nearly as extensive as that observed with the p368 antibody immunoreactivity. This result confirms that S368 phosphorylation, in particular, is increased dramatically via TPA treatment, whereas phosphorylation at many other residues was not as TPA responsive (Lampe et al., 2000), essentially diluting the p368 signal. Furthermore, the total Cx43 signal and the ratio of 'phosphorylated' (i.e. P1 + P2) to nonphosphorylated (Fig. 2, α-368 panel) were quite similar regardless of TPA treatment, in spite of the fact that dramatic changes in S368 phosphorylation occurred.

NRK cells were also labeled with [^{35}S]methionine, and immunoprecipitations were carried out using either the p368 antibody or an anti-Cx43 (Cx43CT1) antibody that shows a strong preference for the NP migratory isoform. These samples were run on SDS-PAGE and analyzed by autoradiography. In NRK cells, the α-Cx43 antibody immunoprecipitated a single band that did not change significantly in intensity upon TPA treatment (Fig. 4, NRK panel, α-Cx43). As expected, this band migrates at the same position as NP. The p368 antibody also immunoprecipitated a single band that migrated exactly with the band immunoprecipitated with the α-Cx43 antibody and showed increased signal intensity upon TPA treatment (Fig. 4, NRK panel, α-p368). Thus, the p368 antibody was able to immunoprecipitate a Cx43 isoform that migrates the same as the NP form in our typical Laemmli gel system, and TPA-treated cells contained more of this isoform than control cells.

Taken together, these metabolic labeling data show that a phosphorylated species of Cx43 essentially co-migrates with the NP form and that this phosphoform can be clearly detected using the p368 antibody. Nonphosphorylated Cx43 and Cx43 phosphorylated at S368 probably could be separated given the appropriate separation technique since these species vary in net charge. It is noteworthy that standard isoelectric focusing and two-dimensional analysis of Cx43 has been shown to be

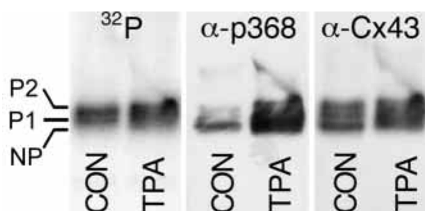

Fig. 3. TPA-treated NRK cells show increased phosphorylation at S368 with no apparent shift in migration in SDS-PAGE. NRK cells were metabolically labeled with [^{32}P]orthophosphate and incubated in the presence (TPA) or absence (CON) of 50 ng/ml TPA for 30 minutes followed by immunoprecipitation of Cx43, blotting to nitrocellulose, and probing the blot first by autoradiography (^{32}P) and then using the anti-p368 antibody (α-p368) and ultimately the N-terminal Cx43 antibody (α-Cx43).

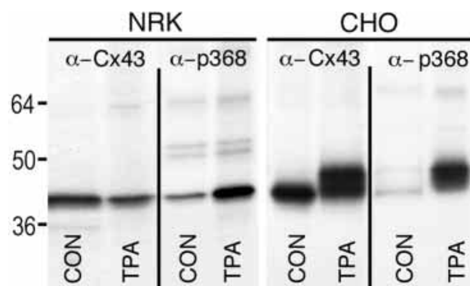

Fig. 4. The p368 antibody immunoprecipitates more Cx43 from either NRK or CHO cells after TPA treatment. NRK and CHO cells were metabolically labeled with [^{35}S]methionine and either treated with TPA or left untreated (CON) and then lysed and immunoprecipitated with either the Cx43 antibody (α-Cx43 lanes) or the p368 antibody (α-p368 lanes).

difficult (Stockert et al., 1999). Nevertheless, when analyzed by a standard Tris/glycine SDS-PAGE system that has been used by most investigators, the migration of Cx43 phosphorylated on S368 often coincided with nonphosphorylated Cx43. For this reason, we believe that it is probably most accurate to refer to the fastest-migrating form as P0 rather than NP when discussing standard SDS-PAGE separation of Cx43 from cells that have been treated with kinase effectors or growth factors, and we do so below. This is an interim solution since different cell types and slightly modified gel systems appear to produce Cx43 with varying migratory properties. A better definition of terms will probably require a thorough understanding of the molecular events that underlie the shift in migration.

The TPA-dependent shift in Cx43 migration, but not phosphorylation of S368, is cell-type specific

Although the migration of Cx43 derived from NRK cells does not shift significantly in the presence of TPA, many other cell types can show a dramatic shift, essentially leaving little faster-migrating species as shown for HeLa cells in Fig. 1. We found that CHO cells also show a dramatic shift in response to TPA, as is shown via immunoprecipitation in Fig. 4 and western immunoblot in Fig. 5 (CHO panels). Fig. 4 shows [^{35}S]methionine-labeled CHO cell lysates immunoprecipitated with α-Cx43 or α-p368 antibodies. In TPA-treated CHO cells, α-Cx43 (Cx43CT1) immunoprecipitated both the NP/P0 migratory isoform and the slower-migrating isoforms (Fig. 4, CHO panel, α-Cx43; see Materials and Methods for antibody description). Immunoprecipitation of TPA-treated CHO cell lysates with α-p368 antibody shows primarily the slower-migrating isoforms (Fig. 4, CHO panel, α-p368) indicating that, in this cell line, phosphorylation on S368 was coincident with a shift in migration.

Similarly, Fig. 5 shows an immunoblot of NRK and CHO whole cell lysates that was probed with the antibody for p368

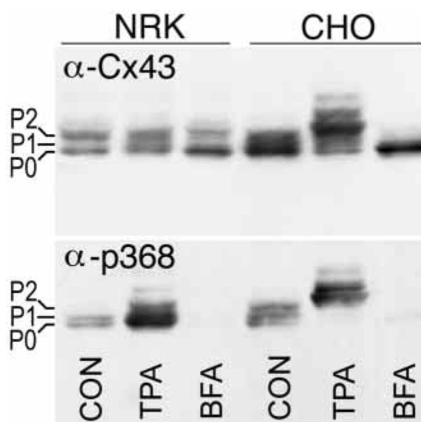

Fig. 5. NRK and CHO cells both show large increases in phosphorylation at S368 upon TPA treatment but the resulting Cx43 mobilities are very different. Cells were either incubated in the presence of no drugs (CON), 50 ng/ml TPA for 30 minutes (TPA) or 5 μg/ml brefeldin A for 4 hours (BFA), and processed for immunoblot and separately probed with the anti-p368 antibody (α-p368) and the N-terminal Cx43 (α-Cx43).

(α-p368) and stripped/reprobed for Cx43 (α-Cx43). Consistent with the immunoprecipitation results, Cx43 from NRK cells did not shift its migration in response to TPA while the protein extensively shifts to slower-migrating phosphoforms in CHO cells. Both cell types show large TPA-dependent increases in reactivity to the p368 antibody, but the p368 signal in NRK cells primarily migrated at the P0 and P1 positions whereas the p368 signal was highly shifted in the CHO cells. HeLa cells containing wt Cx43 (Fig. 1) were intermediate between the two, as p368 is found in the P1 and P2 forms. Notably, in all cell lines examined, a low level of p368 was present in untreated cells and often co-migrated with the NP/P0 isoform, which indicates that phosphorylation of S368 is part of the normal lifecycle of Cx43 in these cells. To examine whether phosphorylation at S368 might be consistent with the early phosphorylation event found in the presence of Brefeldin A (BFA) (Laird et al., 1995), we treated NRK and CHO cell lysates with BFA and found decreased p368 antibody labeling. However, α-p368 binding was not eliminated, so no firm conclusions can be drawn with respect to this event. Thus, we have used the p368 antibody to examine TPA-induced phosphorylation of Cx43 in NRK, CHO and Cx43-transfected HeLa cells and found that all cell types examined show increased phosphorylation on S368, but the degree to which this resulted in a shift in the migration of Cx43 varied between cell types.

TPA-induced phosphorylation of S368 occurs on both intracellular and plasma membrane Cx43

To determine whether a specific pool of Cx43 is phosphorylated in response to TPA, immunofluorescence was performed on NRK cells with an antibody specific for Cx43 (Cx43IF1) and the p368 antibody. NRK cells show extensive immunofluorescence for Cx43 at cell-cell interfaces (Fig. 6, upper left). Upon TPA treatment, Cx43 immunofluorescence showed no apparent change although the cells adopted a slightly more fibroblastic appearance (Fig. 6, lower left). The p368 antibody also showed some cell-cell interface labeling and a light reticulate pattern throughout the cytoplasm (upper center panel). The apparent cytoplasmic pool of p368 staining does appear to be at least partly associated with the endoplasmic reticulum as there was co-localization of p368 with an endoplasmic reticulum-specific dye, R6 (data not shown). After TPA treatment, the p368 signal was greatly increased in both cytoplasmic and interface membranes (lower center panel). The plasma membrane pool of p368 shows co-localization with the Cx43IF1 antibody, whereas less-distinct co-localization of this antibody with the intracellular pool was observed. The increase in intracellular fluorescence does appear to be specific to the p368 epitope as co-incubation of p368 antibody with the peptide antigen used to generate the antibody blocked antibody binding, while co-incubation of the antibody with a nonphosphorylated peptide representing 360-382 of Cx43 did not block binding (data not shown). We have observed that there is competition between Cx43IF1 and p368 antibody binding at cell-cell contacts. This was manifest by a decrease in p368 signal when p368 and Cx43IF1 antibodies were added together, but was reversed by inclusion of the nonphosphorylated peptide, which removed the Cx43IF1 signal.

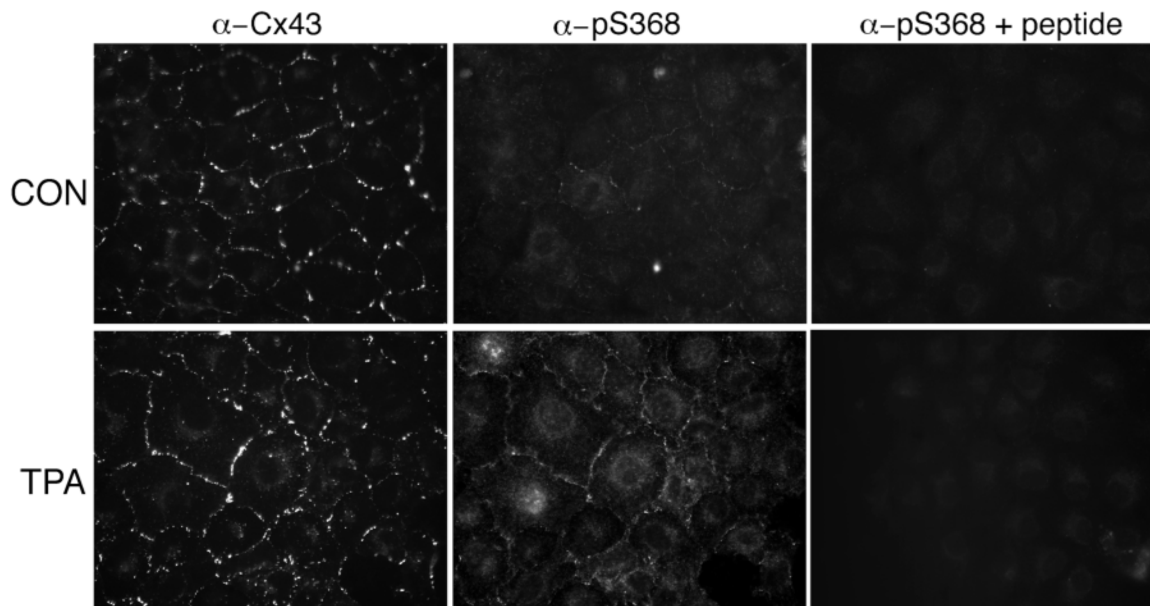

Fig. 6. The p368 antibody binds to both junctional and cytoplasmic membranes. NRK cells that had been incubated in the presence (TPA) of 50 ng/ml TPA or absence (CON) were processed for immunofluorescence with the anti-p368 (α-pS368) and the Cx43IF1 (α-Cx43) antibodies (left and center panels) or with the anti-p368 antibody plus the immunizing peptide (right panel, α-pS368 + peptide).

Phosphorylation on S368 is regulated as cells progress through the cell cycle

Given that phosphorylation on S368 appeared to be part of the normal lifecycle of Cx43, we wanted to determine circumstances under which this event was regulated. As it has previously been shown that Cx43 phosphorylation increases as cells progress through the cell cycle (Kanemitsu et al., 1998), we looked at Cx43 and phosphorylation of S368 in cells synchronized at different stages of the cell cycle in NRK cells. Fig. 7 shows an immunoblot probed first for p368 (α-p368) and then stripped/reprobed for Cx43 (α-Cx43). Vinculin was also detected for a loading control. Densitometry was performed for Cx43 and p368 antibody binding, and the ratio of p368/Cx43 densitometry is shown at the bottom of the figure. Cx43 phosphorylated at S368 was most abundant relative to total Cx43 during S and G_2/M. This result is consistent with previous reports where gap junctional communication was shut-down during mitosis (Stein et al., 1992; Xie et al., 1997) and phosphorylation at S368 had been shown to reduce communication (Lampe et al., 2000). Here, we found that G_0 cells contain very little p368 and that S368 is increasingly phosphorylated as cells approach and progress through S phase.

Given the 7× increase in phosphorylation at S368 when G_0 and S phase cells were compared (Fig. 7), we wanted to examine Cx43 distribution and intercellular communication in these two cell populations. G_0 cells showed strong plasma membrane staining for Cx43 at cell-cell interfaces consistent with gap junctions (Fig. 8A), while S-phase cells showed both typical gap junctional labeling and also extensive perinuclear staining (Fig. 8B). Immunofluorescent labeling with the p368 antibody showed both cytoplasmic and gap junctional staining for both G_0- and S-phase cells (data not shown).

Since TPA treatment of cells has been reported to decrease intercellular communication via changes in channel gating (e.g., Kwak et al., 1995c; Lampe et al., 2000; Moreno et al., 1994) and gap junction assembly (Lampe, 1994), we assessed both the ability to transfer dye and the ability to assemble junctions in G_0- and S-phase cells. When we microinjected G_0- and S-phase cells with two fluorescent dyes of the Alexa series (A488, M_r=570.5; A594, M_r=758.8), we found that S-phase cells transferred both dyes approximately twice as well as G_0 cells (Fig. 8C). However, G_0-phase cells were approximately twice as likely to transfer dye to their neighbors than S-phase

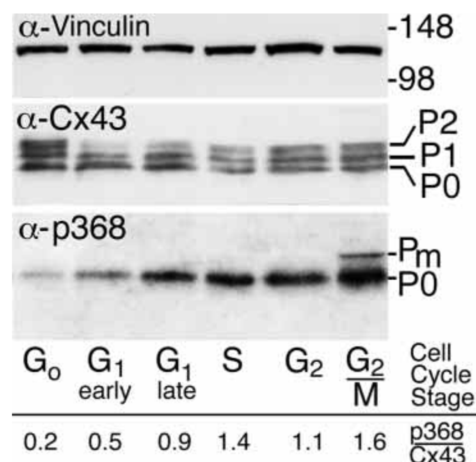

Fig. 7. The extent of p368 phosphorylation is increased through the cell and is maximal during S and G_2/M. Synchronized cells collected at the indicated cell-cycle stage were processed for immunoblotting and probed with antibodies to p368 (α-p368), Cx43NT1 (α-Cx43), or vinculin (for a loading control). The molecular weight or migration position of the Cx43 is indicated on the right, and the ratio of the extent of p368 to Cx43NT1 antibody labeling is shown on the bottom line.

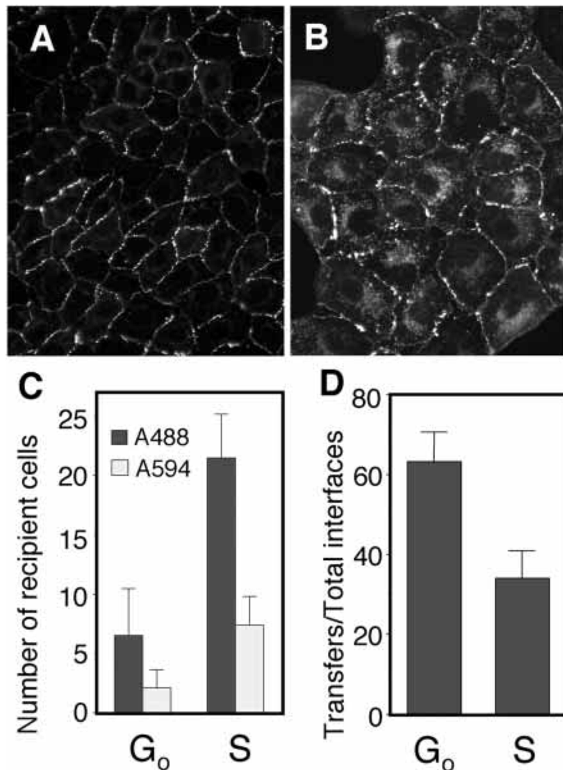

Fig. 8. G_0- and S-phase cells show different Cx43 cellular distributions and different abilities to transfer fluorescent dyes in established and junctional assembly assays. (A) G_0 cells show extensive junctional Cx43 immunostaining. (B) S-phase cells show extensive junctional and cytoplasmic membrane staining. (C) The number of cells that receive either Alexa488 (A488) or Alexa594 (A594) from the injected cell is quantitated for cells in established G_0- or S-phase cultures (mean±s.d.). (D) Cells in G_0 or S were assayed for the ability to assemble gap junctions and transfer calcein (quantitated as the number of transfers per the total number of interfaces between a calcein-loaded and a recipient cell, mean±s.d.).

cells when the calcein/DiI assay, which requires nascent gap junction assembly, was performed (Fig. 8D).

Discussion

Previously, we have demonstrated that phosphorylation of Cx43 on S368 is stimulated by TPA in vivo and mediated by PKC in vitro (Lampe et al., 2000). Furthermore, electrophysiological studies of Cx43 and the Cx43-S368A mutant revealed that phosphorylation at S368 is necessary for a TPA-induced alteration of Cx43 channel behavior that contributes to decreased gap junctional communication. Here, we report that phosphorylation levels at S368 are high in S and G_2/M, and that cells at quiescence show only very low levels. In addition, S-phase cells assembled gap junctions poorly compared with G_0-phase cells, implying a role for phosphorylation at S368 in the regulation of assembly.

Cx43 phosphorylation at S368 appears to occur normally in dividing cells. The seven- to eightfold increase in the level of phosphorylation on S368 at S and G_2/M, respectively, correlates well with increased cytoplasmic localization of Cx43 during S (Fig. 7) and G_2/M (Lampe et al., 1998a; Xie et al., 1997), consistent with a role for S368 phosphorylation in regulating Cx43 trafficking/assembly into gap junctional structures. Interestingly, we also occasionally observed a unique and apparently nuclear envelope/endoplasmic reticulum localization of the p368 antibody at the early stages of G_2/M (data not shown). This immunolocalization was highly transitory because it was lost as the nuclear envelope broke down as the cells entered mitosis. Although this localization appeared specific for the antibody based on antigen competition studies, Cx43IF1 antibody immunolabeling of the nuclear envelope region of G_2/M cells was not nearly as striking as the p368 antibody. Thus, we cannot rule out the possibility that an alternative non-connexin epitope that specifically reacts with the p368 antibody is expressed in early mitosis.

Much of the work examining TPA-mediated downregulation of Cx43 has been motivated by the role of PKC activators as tumor promoters and the potential role of gap junctional communication as a tumor suppressor. Although details are still poorly understood, there is a wealth of data showing that Cx43 phosphorylation is increased and gap junctional communication is reduced upon activation of PKC (reviewed by Lampe and Lau, 2000). However, the use of different assays for communication, several methods for assaying Cx43 phosphorylation and various cellular systems expressing different isoforms of PKC (Cruciani et al., 2001; Munster and Weingart, 1993) have confused the interpretation of the role PKC plays as a modulator of gap junctional communication. For example, several reports have fueled the controversy as to whether mitogen-activated protein kinase (MAPK) or PKC is the actual kinase that phosphorylates Cx43 and reduces gap junctional communication after growth factor or phorbol ester treatment, or whether Cx43 phosphorylation even plays a direct role (e.g., Hossain et al., 1999; Kanemitsu and Lau, 1993; Rivedal and Opsahl, 2001; Vikhamar et al., 1998). One presumption found in many of these reports that might cloud interpretation of the data is that a shift in Cx43 migration has been equated with increased phosphorylation. We know that Cx43 can be phosphorylated at many (>5) sites in untreated cells and at many more sites in growth factor-treated cells (Lampe and Lau, 2000). At this time, we have no understanding of the molecular events responsible for the shift in migration, or of any of the serines involved. By comparing two cell types where TPA led to a shift in Cx43 migration in one but no change in another, the logical but potentially erroneous conclusion could be that Cx43 phosphorylation levels only changed in one of the cell types. For example, from the α-Cx43 panel of Fig. 5, one could conclude that there was a large change in Cx43 phosphorylation in CHO cells upon TPA treatment, whereas NRK cells showed little change and thus appeared unresponsive to TPA treatment; by contrast, the α-p368 panel shows that phosphorylation was dramatically increased at this site in NRK cells. In fact, there probably is some correlation with the extent of shift and the overall level of Cx43 phosphorylation. However, specific phosphorylation events and not the overall level of phosphorylation probably elicit a specific regulatory event such as assembly, disassembly or gating changes. We believe re-evaluation of many of these seemingly conflicting results might be resolved by assaying for TPA and growth factor effects with the p368 and other phosphorylation-site-specific antibodies.

Phosphorylation of Cx43 appears to regulate the trafficking of Cx43 to the plasma membrane, assembly of Cx43 into gap junctional structures, single channel behavior and Cx43 degradation. The latter three events have been reported to be sensitive to TPA and, therefore, could be regulated by PKC (Kanemitsu and Lau, 1993; Kwak et al., 1995a; Kwak et al., 1995c; Lampe, 1994). Our immunofluorescence data with the p368 antibody and comparison of the kinetics of the mobility shift in SDS-PAGE with decreases in gap junctional communication (Kanemitsu and Lau, 1993) indicate that S368 phosphorylation and potentially other PKC-mediated events can occur prior to export to the plasma membrane (Lampe, 1994). Therefore, at least some TPA-dependent phosphorylation at S368 occurs prior to gap junction assembly.

Intercellular communication was reduced by TPA in quiescent but not proliferating NRK cells (Paulson et al., 1994). Data presented here indicates that, in addition to S368 being a TPA-responsive site, there is regulation of S368 phosphorylation during the normal lifespan of Cx43 in untreated, cycling cells. Although S-phase cells transferred dye more rapidly than G_0 cells in established cultures, S-phase cultures were less able to form new functional gap junctions in an assembly assay (Fig. 8D). Clearly, cell-cycle regulation plays a key role during tumorigenesis. The cell-cycle-mediated regulation shown here might indicate a more-subtle and physiological role for gap junctional communication through S368-mediated effects on assembly. Cell-cycle-mediated regulation of Cx43 has been shown during mitosis, when there is a dramatic change in phosphorylation and Cx43 is localized predominately to cytoplasmic membranes. A model in which assembly is most efficient during G_0/G_1, and then decreases as cells progress towards mitosis, this being partially due to phosphorylation at S368, fits our data. During tumorigenesis or faulty regulation of the cell cycle, this decrease in assembly could have dramatic effects on gap junctional communication.

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