

Na-H Exchange-dependent Increase in Intracellular pH Times G₂/M Entry and Transition*

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It is well established that activation of the Na-H exchanger NHE1 and increases in intracellular pH (pH_i) are early and universal responses to mitogens and have permissive effects in promoting cell proliferation. Despite this evidence, a specific role for NHE1 or pH_i in cell cycle progression remains undetermined. We now show that NHE1 activity and pH_i regulate the timing of G₂/M entry and transition. Prior to G₂/M entry there is a rapid and transient increase in NHE1 activity and pH_i, but in fibroblasts expressing a mutant NHE1 that lacks ion translocation activity, this increase in pH_i is attenuated, S phase is delayed, and G₂/M transition is impaired. In the absence of ion translocation by NHE1, expression of cyclin B1 and the kinase activity of Cdc2 are decreased and Wee1 kinase expression increases. Increasing pH_i in the absence of NHE1 activity, however, is sufficient to restore Cdc2 activity and cyclin B1 expression and to promote G₂/M entry and transition. These data indicate that a transient increase in pH_i induced by NHE1 promotes the timing of G₂/M, and they suggest that increases in pH_i at the completion of S phase may constitute a previously unrecognized checkpoint for progression to G₂ and mitosis.

The ubiquitously expressed plasma membrane Na-H exchanger NHE1¹ regulates intracellular pH (pH_i) homeostasis and has a permissive effect in promoting cell proliferation. Activation of NHE1 and increased pH_i are early and universal responses to mitogenic stimulation (1). Growth factor-dependent cell proliferation is attenuated in NHE1-deficient cells (2, 3) in cells treated with pharmacological inhibitors of NHE1 (4–6) and in cells expressing a mutant NHE1 that is deficient in ion translocation (7). Additionally, retrovirus-induced transformation requires an NHE1-dependent increase in pH_i, and clamping pH_i to prevent alkalization inhibits proliferation and a transformed phenotype (8). Despite an established role for NHE1 in mitogen-induced cell proliferation, the mechanisms whereby NHE1 activity and increased pH_i promote cell proliferation are not well understood. We therefore investigated cell cycle progression in fibroblasts expressing a mutant NHE1 that selectively lacks ion translocation activity and is unable to regulate pH_i.

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¹ The abbreviations used are: NHE1, Na-H exchanger isoform 1; pH_i, intracellular pH; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; Cdc2, cyclin-dependent kinase 2; EIPA, ethylisopropylamiloride; FCS, fetal calf serum; HA, hemagglutinin.

EXPERIMENTAL PROCEDURES

Cell Preparation—Ltk⁺ and LAP1 fibroblasts were obtained from J. Pouyssegur (University of Nice, France) (9). LAP1 cells stably expressing wild-type and NHE1-266I were obtained by co-transfection of pRSV-neo (1.0 μg) with NHE1 plasmids (10 μg of pCMV-NHE1) as described previously (7). Cells were maintained in DMEM supplemented with 10% FCS (growth medium).

NHE1 Activity and pH_i—NHE1 activity was determined as described previously (10) in cells loaded with the acetoxymethyl ester derivative of the pH-sensitive dye 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF, Molecular Probes). NHE1 activity was determined by measuring the rate of pH_i recovery (dpH_i/dt) from an NH₄Cl-induced acid load by evaluating the derivative of the slope of the time-dependent pH_i recovery at intervals of 0.05. The pH_i-dependent H⁺ efflux was calculated as the product of dpH_i/dt and the total intracellular buffering capacity (β_i) and expressed as μM/s as described previously (11). Cytosolic pH was determined by measuring BCECF fluorescence in the presence of 25 mM NaHCO₃ to ensure the function of HCO₃⁻-dependent ion exchangers (7). Data represent the mean ± S.E. of four to six separate cell preparations.

Cell Cycle Analysis—DNA content was determined by flow cytometry of cells labeled with propidium iodide and data analyzed by using CellQuest software (BD Biosciences). A double thymidine block was performed by a modification of previously described methods (12). In brief, 24 h after plating cells, 2 mM thymidine was added to the growth medium. After 20 h, cells were washed twice with phosphate-buffered saline and released from the first thymidine block for 9 h in growth medium. A second block was initiated by adding 2 mM thymidine, and cells were maintained for 20 h. Cells were washed in phosphate-buffered saline and released into growth medium for the indicated times. Mitotic index was determined as described previously (13).

Immunoprecipitation, Immunoblotting, and Kinase Activity—Immunoprecipitation and immunoblotting of HA-tagged NHE1 was performed as described previously (7) using anti-HA monoclonal antibody (12CA5; Roche Applied Science). For immunoprecipitation of Cdc2, cells plated in 100-mm dishes were lysed in radioimmune precipitation assay buffer supplemented with 0.1 TIU/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of protein from post-nuclear supernatants (10,000 × g for 5 min) were precleared for 1 h with protein-G-Sepharose (Zymed Laboratories Inc.) and then incubated with anti-Cdc2 antibodies (Santa Cruz Biotechnology) for 2 h followed by protein-G-Sepharose for 1 h. Cdc2 kinase activity was determined by phosphorylation of histone H1 as a substrate in *in vitro* kinase assays using immunoprecipitated Cdc2 as described previously (14). For immunoblotting total Cdc2, PY15-Cdc2, cyclins, and β-actin, equal amounts of protein from post-nuclear supernatants were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with the indicated antibodies (Santa Cruz Biotechnology), and bound antibody was detected by using the enhanced chemiluminescence system (Amersham Biosciences). The protein expression was analyzed by using NIH Image and the abundance of histone H1 phosphorylation was determined by phospho-imager analysis.

TaqMan™ Analysis—TaqMan™ analysis was performed in asynchronous cells using TaqMan™ chemistry with the ABI 7700 Prism real-time PCR instrument (Applied Biosystems, Foster City, CA). The forward and reverse primers for mouse Wee1 kinase were 5'-TTGCTCTTCTCTCACAGTCGT-3' and 5'-TGGGAAAGCACTTGTGGGAT-3', respectively, and the probe was 5'-FAM/CCTTCCAGAAATGAGAGCACTGGC/TAM-3', corresponding to an amplicon of 118 bp. PCR was conducted in triplicate with 50-μl reaction volumes of 1× PCR

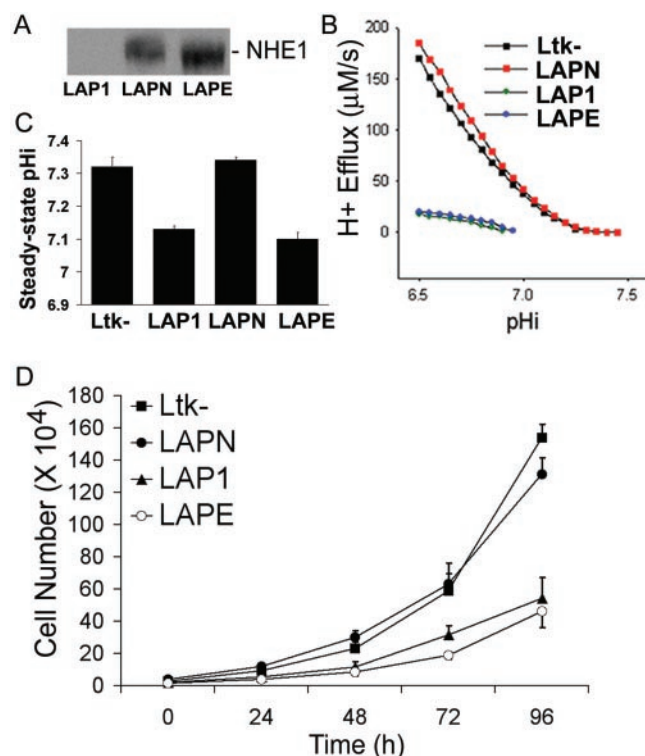


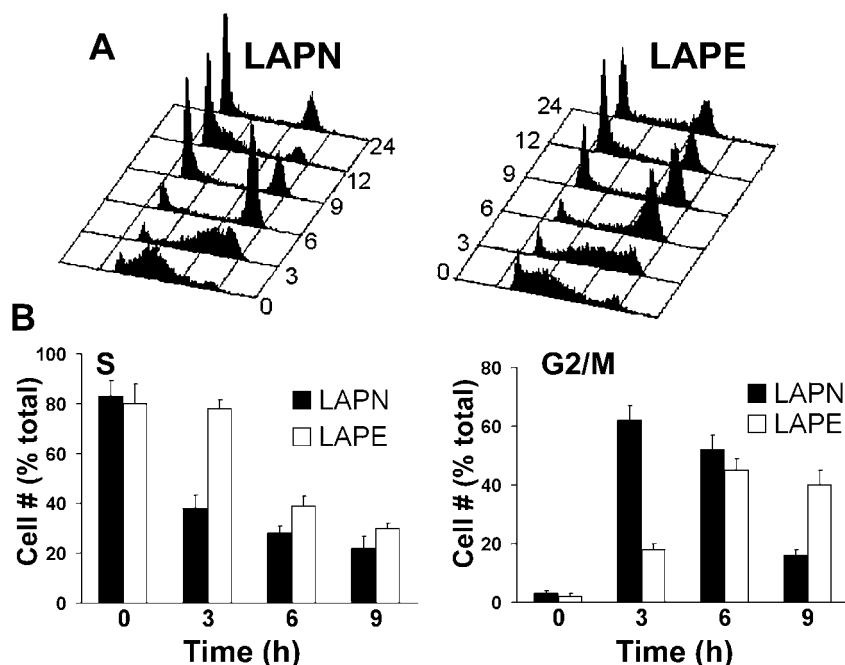
FIG. 1. Ion translocation by NHE1 promotes cell proliferation. A, expression of NHE1, determined by immunoblotting anti-HA immunoprecipitates from total cell lysates obtained from NHE1-deficient LAP1 cells, and LAP1 cells stably expressing HA-tagged wild-type NHE1 (LAPN) or mutant NHE1-E266I (LAPE). B, H⁺ efflux was determined in a nominally HCO₃⁻-free Hepes buffer to confirm that Ltk⁻ and LAPN cells expressing wild-type NHE1, but not NHE1-null LAP1 cells or LAPE cells expressing the mutant NHE1-E266I, have a pH_i-dependent H⁺ efflux. C, steady-state pH_i, determined in the presence of 25 mM NaHCO₃ in cells maintained in continuous 10% FCS, was significantly lower in NHE1-deficient LAP1 cells and in LAPE cells expressing NHE1-E266I than in Ltk⁻ and LAPN cells expressing wild-type NHE1. Data represent the mean ± S.E. of six separate cell preparations. D, proliferation of cells maintained in continuous 10% FCS was decreased in LAP1 and LAPE cells lacking NHE1 activity compared with Ltk⁻ and LAPN cells expressing wild-type NHE1. Data represent the mean ± S.E. of three separate cell preparations.

buffer A (Applied Biosystems), 2.5 mM MgCl₂, 0.4 μM each primer, 200 μM each dNTP, 100 nM probe, and 0.025 unit/μl Taq Gold (Applied Biosystems). For each experiment, a large master mix of the above components was made and aliquoted into each optical reaction tube. The primer/probe set (5–10 μl) was then added and PCR conducted using the following cycle parameters: 95 °C 12 min × 1 cycle (95 °C 20 s, 60 °C 1 min) × 40 cycles. Data analysis was carried out using sequence detection software that calculates the threshold cycle (Ct) for each reaction, which is used to quantitate the amount of starting template in the reaction. A difference in Ct values (ΔCt) was calculated for each gene by taking triplicate Ct values from three reactions and subtracting the mean Ct of the triplicates for the control gene, *GAPDH*, for each cDNA sample at the same concentration. An additional difference in Ct values (ΔΔCt) was calculated for each gene by taking the triplicate ΔCt values for each gene in the mutant LAPN1-E266I cells and subtracting the mean ΔCt of the triplicates for the wild-type LAPN cells. As described previously (15), the relative expression levels were calculated as $2^{-\Delta\Delta Ct}$.

RESULTS AND DISCUSSION

Recent evidence indicates that in addition to its function in ion translocation and pH_i homeostasis, NHE1 also acts as a scaffold to assemble signaling complexes (1, 15) and as a plasma membrane anchor for the actin-based cytoskeleton (7, 16, 17). To selectively impair only the ion translocation function of NHE1, we engineered an ion translocation-defective NHE1 containing an isoleucine substitution for glutamine 266 (NHE1-E266I). In cells expressing NHE1-E266I, the scaffolding and actin anchoring functions of NHE1 are retained, but ion translocation is absent (7, 16, 17). For the current study, the effect of ion translocation by NHE1 on cell proliferation was assessed by using NHE1-null LAP1 fibroblasts (9) selected to stably express either wild-type NHE1 with a C-terminal HA tag (LAPN cells) or HA-tagged NHE1-E266I (LAPE cells). Expression of NHE1 in LAPE cells is slightly greater than that in LAPN cells (Fig. 1A), and the increased gel mobility of NHE1-E266I likely reflects a difference in glycosylation (18). NHE1-null LAP1 cells are derived from parental Ltk⁻ cells expressing endogenous NHE (9), and Ltk⁻ cells have a pH-dependent H⁺ efflux that is not observed in LAP1 cells (Fig. 1B). Stable expression of wild-type NHE1 in LAPN cells, but not NHE1-E266I in LAPE cells, restored this efflux (Fig. 1B). Moreover, in LAP1 and LAPE cells maintained in 10% FCS and the presence of 25 mM NaHCO₃ and 5% CO₂ to ensure the function of HCO₃⁻-dependent transporters, steady-state pH_i (Fig. 1C) and

FIG. 2. NHE1 activity promotes G₂/M entry. A, DNA content of LAPN and LAPE cells at the indicated times after release from a double thymidine block was analyzed by flow cytometry. B, the percentage of LAPN and LAPE cells in S phase (left) and G₂/M (right) at the indicated times after release from a double thymidine block. Data represent the mean ± S.E. of three separate cell preparations.



the rate of cell proliferation (Fig. 1D) were significantly lower than that in Ltk⁻ and LAPN cells. These findings are consistent with those of previous reports (3, 7) that NHE1 activity provides a permissive signal for cell proliferation.

An initial analysis of DNA content in asynchronous cells indicated that G₂/M progression was delayed in LAPE cells compared with LAPN cells (data not shown). This was further investigated by arresting cells in S phase with a double thymidine block and analyzing DNA content by flow cytometry at the indicated times after release from arrest (Fig. 2A). At the time of release (time 0), 83 ± 6% of LAPN cells and 80 ± 8% of LAPE cells were in S phase (mean ± S.E.; *n* = 3 cell preparations) (Fig. 2B). At 3 h after release, 55 ± 5% of LAPN cells, but only 18 ± 2% of LAPE cells, had entered G₂/M (Fig. 2B). At 6 h after release, 58 ± 5% of LAPN cells and 47 ± 4% of LAPE cells were in G₂/M, and 22 ± 3% of LAPN cells and 39 ± 4% of LAPE cells remained in S phase. Hence, in the absence of NHE1 activity

there is a delay in S phase and impaired entry into G₂/M. In three separate cell preparations, there was no indication that LAPE cells had increased necrosis or apoptosis compared with LAPN cells.

In LAPN cells, the transition from S phase to G₂/M was associated with a significant increase in p*H*_i and NHE1 activity. Cytosolic p*H*_i, determined in the presence of NaHCO₃, increased steadily after release from a double thymidine block, reaching a maximum of 7.48 ± 0.01 at 3 h (Fig. 3A). At 4 h after release, p*H*_i was rapidly and significantly reduced to 7.26 ± 0.02 (*p* < 0.01) and remained constant for up to 9 h. Additionally, the increase in p*H*_i in LAPN cells at 3 h was associated with a transient increase in NHE1 activity (Fig. 3B), as determined by the rate of p*H*_i recovery (dp*H*_i/dt) from an NH₄Cl-induced acid load in a Hepes buffer. In the absence of NHE1 activity in LAPE cells there was also a time-dependent increase in p*H*_i indicating that H⁺ extrusion mechanisms, independent of NHE1 activity, were activated. The maximum p*H*_i of 7.27 ± 0.01 in LAPE cells, however, was significantly lower than that in LAPN cells (Fig. 3A). These data indicate that the transition from S phase to G₂/M is associated with an increase in NHE1 activity, which promotes a marked and transient increase in p*H*_i. In the absence of NHE1 activity, the p*H*_i increase is attenuated, S phase is delayed, and entry into G₂/M is impaired.

In LAPE cells G₂/M transition was also impaired and the activity and expression of G₂/M regulatory proteins were markedly different compared with LAPN cells. In asynchronous cells, the mitotic index of LAPE cells was 50% lower than that of LAPN cells (4 versus 8%). In eukaryotic cells, mitotic entry is dependent on increased activity of the cyclin-dependent kinase Cdc2 (19). At 3 and 6 h after thymidine release, Cdc2 kinase activity in LAPN cells increased, as determined by phosphorylation of histone H1 in *in vitro* kinase assays using immunoprecipitated Cdc2 (Fig. 4A, top row). Cdc2 kinase activity also increased in LAPE cells, but at delayed 6- and 9-h time points and maximum activity was reduced by 40% compared with LAPN cells (Fig. 4B). The abundance of Cdc2 in LAPN and LAPE cells, however, was similar at all time points (Fig. 4A,

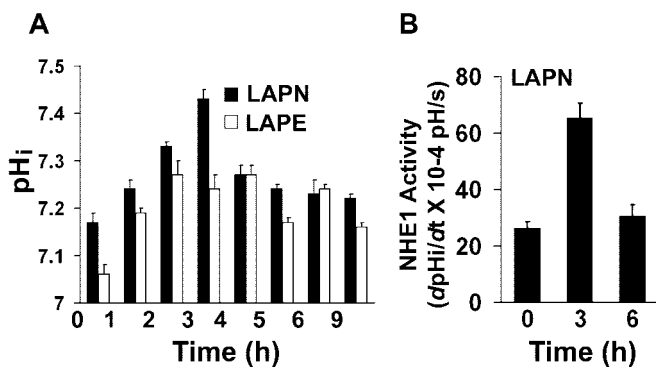
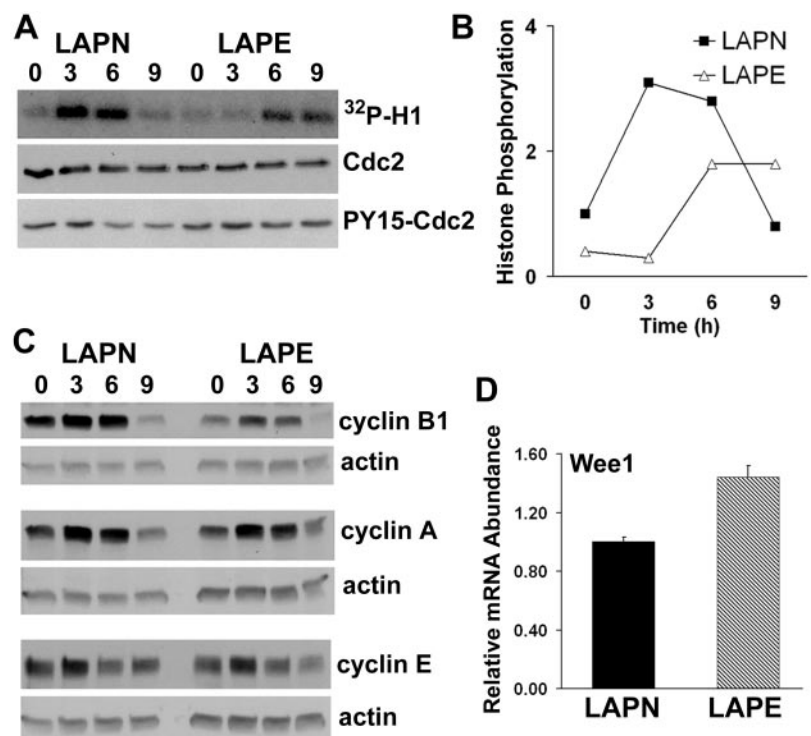


FIG. 3. S phase completion is associated with transient increases in p*H*_i and NHE1 activity. A, cytosolic p*H*_i in LAPN and LAPE cells at the indicated times after release from a double thymidine block. Data represent the mean ± S.E. of four separate cell preparations. B, NHE1 activity in LAPN cells at the indicated times after release from a double thymidine block, as determined by the p*H*_i-dependent rate of p*H*_i recovery (dp*H*_i/dt) from an NH₄Cl-induced acid load. Data are expressed as the mean ± S.E. of NHE1 activity at p*H*_i 6.5, as determined in three separate cell preparations.

FIG. 4. NHE1 activity regulates the activity and expression of G₂/M regulatory proteins. A, Cdc2 kinase activity at the indicated times after release from a double thymidine block was determined by phosphorylation of histone H1 (top row) in *in vitro* kinase assays with Cdc2 immune complexes. Total Cdc2 (middle row) and PY15 Cdc2 (bottom row) were determined at the indicated times after release from a double thymidine block by immunoblotting. B, relative abundance of phosphorylated histone in Cdc2 kinase assays in LAPN and LAPE cells at the indicated times after release from a double thymidine block. Data represent the means of three separate cell preparations. C, abundance of cyclins B1, A, and E in LAPN and LAPE cells at the indicated times after release from a double thymidine block was determined by immunoblotting. Membranes were reprobed for β-actin, and data are representative of three separate cell preparations. D, relative abundance of Wee1 kinase mRNA in asynchronous LAPN and LAPE cells, as determined by TaqMan™ analysis. Data represent the mean ± S.E. of three separate cell preparations.



middle row). Cdc2 activity is regulated in part by its association with the M phase cyclin B1, which begins to be synthesized at the end of S phase (20). In both LAPN and LAPE cells the expression of cyclin B1 increased at 3 and 6 h, however, the abundance of cyclin B1 at all time points was markedly less in LAPE cells (Fig. 4C). In contrast, the abundance of cyclin A (Fig. 4C), cyclin E (Fig. 4C), and cyclin D1 (data not shown) was similar in both cell types at all time points. Cdc2 kinase activity is also regulated by inhibitory phosphorylation on tyrosine 15 (21, 22), which is promoted by the Wee1/Mik1 family of kinases (23, 24). Immunoblotting with a phospho-specific antibody indicated that Tyr¹⁵ phosphorylation of Cdc2 was sustained longer in LAPE cells compared with LAPN cells (Fig. 4A, bottom row), which is consistent with decreased Cdc2 kinase activity. In cDNA array analyses of mRNA isolated from asynchronous cells, LAPE cells had a 2.3-fold increase in Wee1 kinase expression compared with LAPN cells.² This finding was confirmed by TaqManTM analysis, which revealed that Wee1 mRNA was significantly more abundant in asynchronous LAPE cells than in asynchronous LAPN cells (Fig. 4D; $p < 0.05$; $n = 3$ separate cell preparations). Collectively, these data suggest that ion translocation by NHE1 alters the activity and expression of M phase regulators and promotes mitotic entry.

Increased pH_i resulting from increased NHE1 activity could promote G₂/M entry and progression, or alternatively increased pH_i might simply result from G₂/M entry and progression, and because S phase is delayed in LAPE cells, accompanying increases in pH_i are attenuated. In support of pH_i regulating the timing of G₂/M, increasing pH_i in the absence of NHE1 activity was sufficient to rescue the time-dependent entry into G₂/M and the activity and expression of M phase regulators. To confirm that impaired G₂/M entry and transition were not merely due to the constitutive loss of NHE1 activity in LAPE cells, but could also result from acute inhibition of NHE1, the effects of pH_i were determined in LAPN cells treated with the amiloride analog ethylisopropylamiloride (EIPA), which selectively inhibits NHE1 activity (25). At the time of release from a double thymidine block, LAPN cells were treated with either Me₂SO (control) or EIPA (25 μ M) and were maintained in the continuous presence of 25 mM NaHCO₃ and 5% CO₂. At 3 h after release, the pH_i of LAPN treated with EIPA was significantly lower than control cells (Fig. 5A; $p < 0.01$; $n = 4$) and NHE1 activity was completely inhibited (data not shown). To increase pH_i in the absence of NHE1 activity, EIPA-treated LAPN cells were incubated in 50 mM NaHCO₃ at 15% CO₂ at the time of thymidine release, which resulted in an alkalization to 7.38 ± 0.03 , compared with a pH_i of 6.98 ± 0.02 with EIPA alone (Fig. 5A). Moreover, increasing pH_i in EIPA-treated cells was associated with an increased number of cells in G₂/M at 3 h (Fig. 5A). At 3 h after release, the percentage of cells in G₂/M was 56 ± 3 for control LAPN cells, 21 ± 3 for LAPN cells treated with EIPA, and 50 ± 4 for LAPN cells treated with EIPA but maintained in 50 mM NaHCO₃ at 15% CO₂. There was a similarity in the percentage of cells in G₂/M at 3 h in LAPE cells expressing a mutant NHE1 lacking ion translocation (Fig. 2B) and in LAPN cells treated with EIPA (Fig. 5B). Like LAPE cells lacking NHE1 ion translocation, acutely inhibiting NHE1 activity in LAPN cells with EIPA was associated with decreased Cdc2 kinase activity compared with control cells, as indicated by histone H1 phosphorylation by immunoprecipitated Cdc2 (Fig. 5, C and D). Compared with control cells, EIPA treatment also increased Tyr¹⁵ phosphorylation of Cdc2 and decreased cyclin B1 expression (Fig. 5E), indicating that impaired Cdc2 activity and cyclin B1 expression

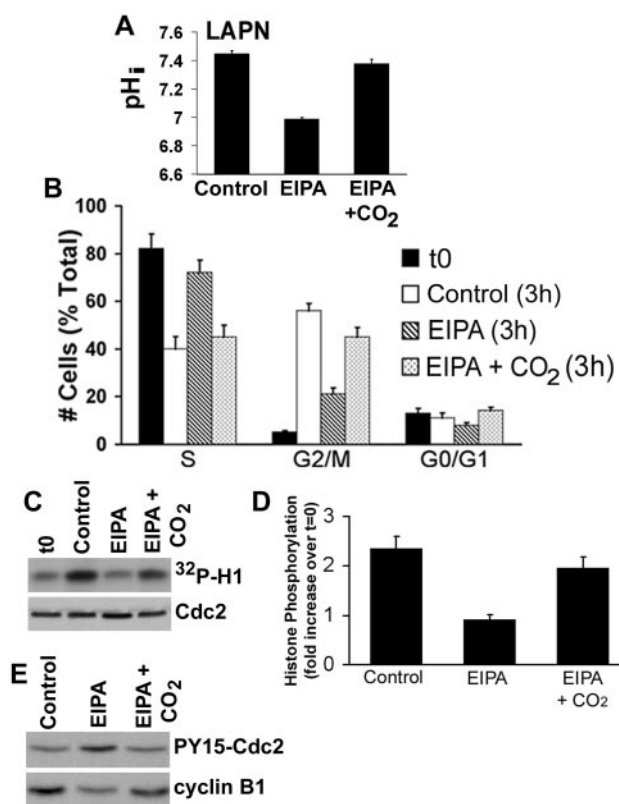


FIG. 5. Increasing pH_i in the absence of NHE1 activity regulates G₂/M timing and regulatory proteins. A, cytosolic pH_i in LAPN cells at 3 h after release from a double thymidine block. At the time of thymidine release, LAPN cells were treated with Me₂SO and maintained in 25 mM NaHCO₃ at 5% CO₂ (Control), treated with 25 μ M EIPA, and maintained in 25 mM NaHCO₃ at 5% CO₂ (EIPA) or treated with 25 μ M EIPA and maintained in 50 mM NaHCO₃ at 15% CO₂ (EIPA + CO₂). Data represent the mean \pm S.E. of four separate cell preparations. B, the percentage of cells in S, G₂/M, and G₀/G₁ at time 0 (t0) and 3 h after release from a double thymidine block, as determined by flow cytometry analysis. Cell conditions were similar to those described in A, and data represent the mean \pm S.E. of three separate preparations. C and D, Cdc2 kinase activity, determined by phosphorylation of histone H1 as described in the legend to Fig. 3A, and abundance of total Cdc2, determined by immunoblotting, at the time of thymidine release (t0) and 3 h after release in Me₂SO-treated LAPN cells (Control) and in LAPN cells treated with EIPA. E, abundance of PY15-Cdc2 and cyclin B1 in Me₂SO-treated LAPN cells (Control) and in LAPN cells treated with EIPA at 3 h after release from a double thymidine block.

were not merely a consequence of constitutive loss of NHE1 activity in LAPE cells but also resulted from acute inhibition of NHE1 activity. Moreover, increasing pH_i in the absence of NHE1 activity was sufficient to increase Cdc2 activity (Fig. 5, C and D), attenuate Tyr¹⁵ phosphorylation of Cdc2 (Fig. 5E), and rescue cyclin B1 expression (Fig. 5E).

Together, our findings suggest that increases in pH_i promote the timing of G₂/M entry and transition and that efficient timing requires a pH_i of greater than 7.30 (see Fig. 3A), which is promoted by NHE1 activity. NHE1 contains an H⁺ modifier site, which renders activity of the exchanger exquisitely sensitive to changes in pH_i . Hence, the transient increase in NHE1 activity could be a response to increased glycolysis and metabolic acid production during DNA replication in S phase, with the concomitant increase in pH_i providing a signal for efficient progression from S phase to G₂/M. Whether the kinase activities of Cdc2 and Wee1 are pH-sensitive is undetermined. It also remains to be determined whether a specific G₂/M regulatory protein is the primary target of increased pH_i , although cyclin B1 is a likely candidate. Cyclin B1 synthesis increases at the

² L. K. Putney and D. L. Barber, manuscript in preparation.

end of S phase (20), and cyclin B1 transcription or mRNA stability could be regulated by pH_i . Additionally, cyclin B1 levels are transiently reduced by DNA damage (12), and decreased pH_i could be part of a DNA damage-sensing response. An important future direction will be to determine whether increasing cyclin B1 expression is sufficient to rescue the delayed timing of G₂/M transition in the absence of transiently increased NHE1 activity and pH_i . Most intriguing is the possibility that transient increases in pH_i at the completion of S phase constitute a previously unrecognized component of a checkpoint pathway.

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