

# Human kidney anion exchanger 1 localisation in MDCK cells is controlled by the phosphorylation status of two critical tyrosines

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## Summary

An important question in renal physiology is how the  $\alpha$ -intercalated cells of the kidney regulate the distribution of the basolateral kidney anion exchanger 1 (kAE1) according to systemic acid-base status. Previous work using a MDCKI model system demonstrated that kAE1 basolateral targeting requires an N-terminal determinant and a critical C-terminal tyrosine (Y904). Here, we show that the N-terminal determinant is residue Y359, because a Y359A substitution mutant was mistargeted to the apical membrane. Further determinants might exist because a range of N-terminal kAE1 truncations that contained Y359 were incorrectly targeted to the TGN. Y359 and Y904 in kAE1 are phosphorylated upon pervanadate treatment and this phosphorylation is sensitive to specific Src kinase family inhibitors. We tested a range of stimuli on this model system and only the application of high nonphysiological

concentrations of extracellular bicarbonate, and to a lesser extent hypertonicity or hyperosmolarity, induced tyrosine phosphorylation of kAE1. Treatment with pervanadate caused internalisation of kAE1 from the plasma membrane, but treatment with high concentrations of bicarbonate did not, because of the hypertonicity of the solution. We propose that  $\alpha$ -intercalated cells control the distribution of kAE1 by reversible phosphorylation of tyrosine residues Y359 and Y904.

Supplementary material available online at  
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Key words: Basolateral trafficking, Kidney anion exchanger 1 (kAE1), Phosphorylation, Tyrosine, Tyrosine motif

## Introduction

The intercalated cells (ICs) of the kidney are classic examples of polarised cells, with well defined apical and basolateral domains containing proton pumps and bicarbonate transporters inserted in opposing membranes. At least two types of ICs exist ( $\alpha$  and  $\beta$ ), which have an opposite configuration of transporter localisation reflecting their different roles in the kidney. The  $\alpha$ -intercalated cells secrete acid by the co-operative action of an apical  $H^+$ -ATPase and the basolateral  $Cl^-/HCO_3^-$  exchanger kidney anion exchanger 1 (kAE1; also known as SLC4A). The apical  $H^+$ -ATPase of the  $\alpha$ -IC pumps protons derived from hydrated  $CO_2$  into the tubular lumen, whereas kAE1 reclaims the remaining bicarbonate back into the blood in exchange for chloride. The bicarbonate secreting  $\beta$ -ICs have an opposing polarity with an apical  $Cl^-/HCO_3^-$  exchanger (pendrin, also known as SLC26A4) and a basolateral  $H^+$ -ATPase to enable net bicarbonate secretion (Wall, 2005).

The human kAE1 present in the basolateral membrane of  $\alpha$ -IC, is an isoform of erythrocyte AE1 (eAE1) truncated at the N-terminus by 65 amino acids (Kollert-Jons et al., 1993). The absence of AE1 through the occurrence of natural mutations in humans (Ribeiro et al., 2000) and cattle (Inaba et al., 1996) or by gene knockouts in mice (Peters et al., 1996) causes distal renal tubular acidosis (dRTA). dRTA is characterised by an inability to acidify the urine, which leads to metabolic acidosis, nephrocalcinosis and eventually kidney stones (Laing et al., 2005). There are increasing numbers of dominant and recessive mutations of AE1 that cause dRTA because of mistargeting of kAE1 away from the basolateral membrane (Cordat et al., 2006;

Cordat and Reithmeier, 2006; Devonald et al., 2003; Rungroj et al., 2004; Toye et al., 2004).

Despite the importance of kAE1 for acid-base homeostasis, very little is known about its trafficking or regulation in the kidney. Animal studies suggest that ICs might increase kAE1 levels at the basolateral membrane during chronic metabolic acidosis and reduce kAE1 levels during acute metabolic alkalosis (Fejes-Toth et al., 1994; Huber et al., 1999; Sabolic et al., 1997; Verlander et al., 1994) but the mechanism for how this occurs is unknown.

AE1 is a 911 amino acid multispanning membrane protein, with both N-terminal (residues 1-359) and C-terminal (residues 881-911) domains located in the cytoplasm. Both the N and C termini are essential for basolateral trafficking of kAE1 (Toye et al., 2004). Several motifs located within the cytosolic domains of integral membrane proteins are involved in the targeting of basolateral proteins, including tyrosine motifs (Yxx $\Phi$ ) and di-leucine motifs (Bonifacino and Traub, 2003). The study of a natural C-terminal truncation mutant kAE1R901stop (Karet et al., 1998; Toye et al., 2002) showed that the C-terminus contains a nonclassical tyrosine motif, Y904 (using erythrocyte AE1 numbering) that is critical for basolateral trafficking (Devonald et al., 2003; Toye et al., 2004; Toye et al., 2002). The identity of the N-terminal trafficking determinant is unknown, but one suggestion is that the N-terminal tyrosine residue Y359 might be important (Toye, 2005; Toye et al., 2004). This is because both Y359 and Y904 are known to be phosphorylated in erythrocyte AE1 (Yannoukakos et al., 1991) and tyrosine phosphorylation may influence membrane protein trafficking by regulating interactions with the cellular targeting

and/or internalisation machinery (Anderson et al., 2005; Bradshaw et al., 1997; Quick et al., 2004; Stephens and Banting, 1997; Sundberg et al., 2004).

Here we investigate the contribution of tyrosine Y359 and the remainder of the N-terminus in polarised kAE1 trafficking. We found that Y359 is an N-terminal determinant critical for AE1 basolateral trafficking, confirming that kAE1 localisation is uniquely controlled by the interplay of two tyrosines, Y359 in the N-terminus and Y904 in the C-terminus. N-terminal truncations of kAE1 were not well tolerated, and the majority were localised to the trans-Golgi network (TGN) at steady state, even though these constructs contain Y359. Both Y359 and Y904 are phosphorylated upon treatment with the phosphatase inhibitor pervanadate or exposure to high nonphysiological concentrations of extracellular bicarbonate, probably by a Src family kinase. We show that treatment with pervanadate resulted in internalisation of normal kAE1 from the plasma membrane, but treatment with high concentrations of bicarbonate did not induce internalisation due to the known disruptive effects of hypertonic solutions on internalisation machinery (Hansen et al., 1993). We propose a mechanism where phosphorylation of the N-terminal and C-terminal tyrosine residues regulates the presence of kAE1 in the basolateral membrane in IC according to the systemic acid-base status.

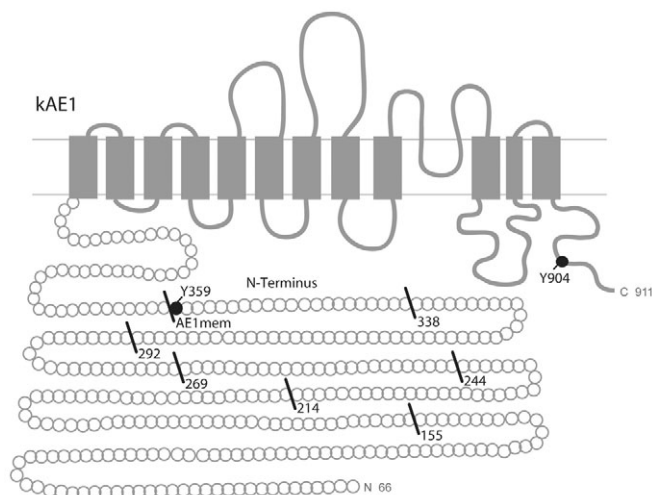
## Results

**An N-terminal domain tyrosine (Y359) is critical for basolateral trafficking**

To establish whether the N-terminal tyrosine residue Y359 is important for basolateral trafficking, we produced a kAE1 construct with tyrosine Y359 substituted by alanine (kAE1-Y359A) and also generated a series of N-terminal deletion constructs designed to identify the minimum length of the kAE1 N-terminus needed for correct trafficking (Fig. 1). We then isolated MDCKI stable cell lines expressing kAE1-Y359A or various N-terminally truncated forms of kAE1.

Fig. 2 shows that wild-type kAE1 is targeted to the plasma membrane in nonpolarised cells (Fig. 2a) and to the basolateral membrane of polarised cells (Fig. 2c) as previously reported (Toye et al., 2004). In comparison, the Y359A substitution resulted in a kAE1 protein that successfully reaches the plasma membrane of nonpolarised MDCKI cells (Fig. 2b), but is mis-sorted to the apical plasma membrane in polarised cells (Fig. 2g). The number of cells expressing kAE1-Y359A was low, precluding biochemical confirmation of the apical targeting. Instead, the exclusive apical localisation of kAE1-Y359A was confirmed by incubating the cells with an extracellular AE1 antibody FITC-Bric6 (Toye et al., 2004) as shown in Fig. 2j. Since kAE1-Y359A is identical to the wild-type protein, except for the Y359A substitution, this result suggests that Y359 is crucial for kAE1 localisation or for its stability at the basolateral membrane.

Fig. 3 shows that only one N-terminal truncation, NtΔ1-155AE1 (Fig. 3e), reached the plasma membrane in nonpolarised cells, whereas three of the shorter truncations tested NtΔ1-338AE1, NtΔ1-292AE1 and NtΔ1-269AE1 (Fig. 3b,c,d, respectively) dramatically altered steady state kAE1 localisation away from the plasma membrane to a perinuclear compartment. The intracellular localisation of NtΔ1-338AE1, NtΔ1-292AE1 and NtΔ1-269AE1 was maintained when cells were polarised, whereas NtΔ1-155AE1 was correctly targeted to the basolateral membrane (results not shown). The compartment coimmunolabelled with TGN38 (Fig. 3k), a well characterised TGN marker (Luzio et al., 1990) and Furin, another TGN marker, but not the ER marker calnexin or late



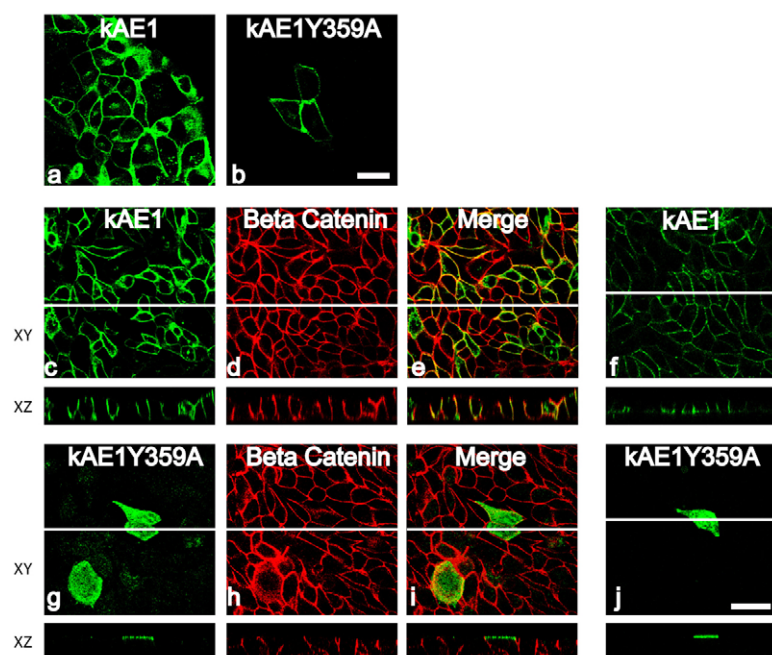
**Fig. 1.** Location of Y359 and Y904 and N-terminal truncation constructs on kAE1. Diagram of human kAE1 indicating the location of N-terminal tyrosine residue Y359, the locations of the N-terminal truncations and C-terminal tyrosine Y904. This illustration is adapted from a previous paper (Williamson and Toye, 2008).

endosomal marker LAMP-1 (supplementary material Fig. S3f,l,i respectively). This observation was surprising because the AE1mem construct (NtΔ1-360AE1) which lacks Y359, can reach the plasma membrane in nonpolarised cells (Fig. 3a). The kAE1 sequence around Y359 (SFY359KGL) is similar to the TGN38 localisation motif SxYxxL, a highly efficient plasma membrane internalisation motif that targets the most of TGN38 to the TGN at steady state (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). However, mutagenesis of the Y359 to an alanine residue in NtΔ338AE1 did not alleviate the intracellular targeting of this construct (Fig. 3f), unlike TGN38, where mutation of the tyrosine in SxYxxL resulted in TGN38 localisation shifting to the plasma membrane (Humphrey et al., 1993; Wong and Hong, 1993). We transiently expressed two additional N-terminal constructs NtΔ1-244AE1 and NtΔ1-214AE1 (Fig. 3g,h) which also had a TGN localisation in the majority of transfected MDCKI cells. Since both Y359 and Y904 are present in all of the N-terminal truncation constructs that were mislocalised to the TGN (between NtΔ1-155AE1 and NtΔ1-360AE1), the inclusion of an incomplete N-terminus must disrupt kAE1 trafficking in some unknown way, for example, by increasing aggregation of the protein.

**kAE1 residues Y359 and Y904 are phosphorylated after pervanadate treatment and this phosphorylation is sensitive to Src kinase inhibitors**

To determine whether kAE1 was phosphorylated in MDCKI-kAE1 on residues Y359 or Y904, we generated phosphospecific antibodies against phosphorylated Y359 (anti-Y359-P) or Y904 (anti-Y904-P) as outlined in the Materials and Methods. These anti-human AE1 phosphotyrosine-specific antibodies were highly specific for human phosphorylated Y359 or Y904 (supplementary material Fig. S1) and did not detect the equivalent phosphorylated tyrosines from the mouse (results not shown). The rabbit antibody raised against the whole cytosolic C-terminus (residues 881-911) of AE1 (anti-AE1Ct) was phosphorylation independent (supplementary material Fig. S1).

We conducted a time course of kAE1 phosphorylation in the presence of pervanadate. Fig. 4A shows that a time-dependent



**Fig. 2.** KAE1-Y359A is targeted to the plasma membrane in nonpolarised MDCKI cells and mistargeted to the apical membrane in polarised MDCKI cells. MDCKI cells stably expressing kAE1 or kAE1-Y359A were seeded on coverslips for nonpolarised cells (a,b) or polarised on filters (c-j). (a-c,g) kAE1 was detected using AE1 monoclonal antibody Bric170. (d,h) Rabbit anti- $\beta$ -catenin antibody was used as a basolateral marker. The corresponding merged images for c and d, and g and h are shown in e and i, respectively. (f,j) An extracellular anti-AE1 antibody FITC-Bric6 was added to both the apical and basolateral surfaces of the intact cell monolayers. For the polarised images, the upper micrographs are focal planes taken parallel to the epithelium (X-Y) through the centre for kAE1 or the apical surface for kAE1-Y359A. The lower micrographs show focal planes perpendicular to the epithelium (X-Z) along the white line in the X-Y image. Normal kAE1 is at the plasma membrane in nonpolarised cells (a) and basolateral membrane in polarised cells using Bric170 (c) or FITC-Bric6 (f). The kAE1-Y359A protein is also at the plasma membrane in nonpolarised cells (b) but mistargeted to the apical membrane using Bric170 (g) or FITC-Bric6 (j). Scale bars: 30  $\mu$ m.

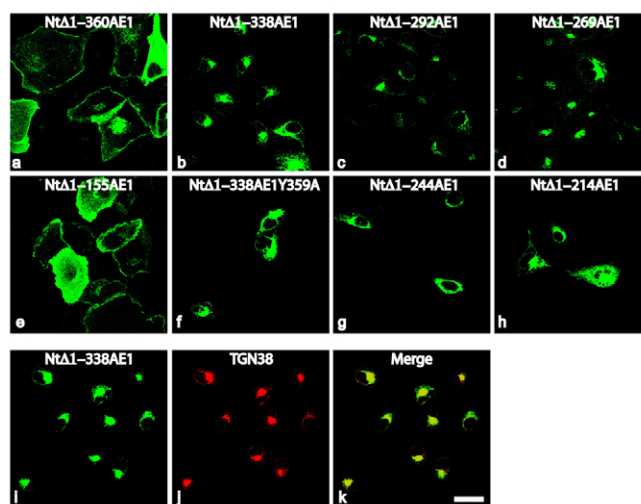
increase in kAE1 phosphorylation occurs. A low level of phosphorylation was evident within 1 minute of incubation with pervanadate and the majority of Y359 and Y904 was phosphorylated within 5 minutes of pervanadate treatment. The time course of phosphorylation seen with pervanadate appears to be similar for both Y359 and Y904 (see supplementary material Fig. S4). Control MDCKI cells or MDCKI-kAE1 cells without pervanadate treatment exhibited no Y359-P or Y904-P signal.

Erythroid AE1 is tyrosine phosphorylated under various conditions (Bordin et al., 2002; Brunati et al., 2000; Harrison et al., 1994; Minetti et al., 1996; Minetti et al., 1998) and a proportion of this phosphorylation is inhibitable by the Src-family-specific inhibitor PP1 (Brunati et al., 2000) and the related inhibitor PP2 (Bordin et al., 2002). Fig. 4B shows that phosphorylation of kAE1 is inhibited after 5 minutes of treatment with pervanadate to a similar level by either 25  $\mu$ M PP1 or PP2. In addition, 25  $\mu$ M SU6656 (another Src-family-specific inhibitor (Blake et al., 2000) or 2  $\mu$ M staurosporine treatment also inhibited kAE1 Y359 and Y904 phosphorylation, but not as potently as PP1 or PP2. The inactive analogue PP3 and the broad-spectrum Src kinase inhibitors genistein and herbimycin did not inhibit kAE1 phosphorylation. Although PP1, PP2 and SU6656 inhibited kAE1 phosphorylation during a short (5 minute) pervanadate treatment, inhibition by these compounds was incomplete. After 5 minutes, pervanadate-induced kAE1 phosphorylation continued in the presence of these inhibitors and approached the level of phosphorylation seen for uninhibited kAE1 after 30 minutes exposure to pervanadate (results not shown). Hence, Src family inhibitor compounds are either inefficient inhibitors of the kinases responsible for kAE1 phosphorylation during pervanadate treatment or non-Src family kinases contribute to kAE1 phosphorylation in the presence of pervanadate.

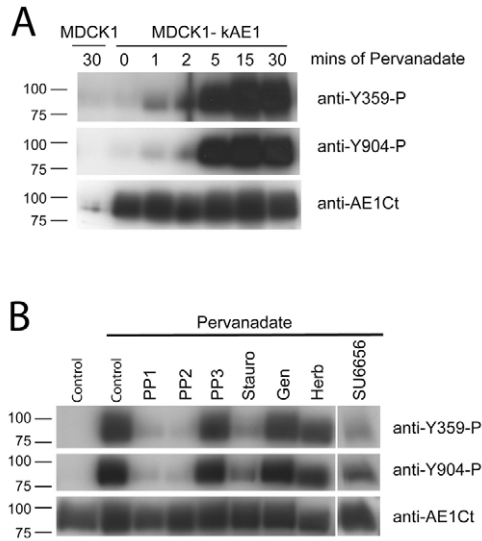
**Hypertonicity, hyperosmolarity or high concentrations of sodium bicarbonate stimulate kAE1 tyrosine phosphorylation in MDCKI cells**

Since kAE1 localisation appears to be influenced by acidosis or alkalosis in the animal models (Huber et al., 1999; Sabolic et al.,

1997), we tested the effects of lowering the pH of the medium (mimicking acidosis) or raising pH using  $\text{NaHCO}_3$  (mimicking alkalosis) on kAE1 tyrosine phosphorylation (Fig. 5A). We found that pH 8.7 medium (corresponding to the addition of 514 mM  $\text{NaHCO}_3$ ) consistently ( $n=25$ ) triggered substantial kAE1 phosphorylation on Y359 (Fig. 5A, lane 5) and to a lesser extent



**Fig. 3.** The majority of N-terminal truncations of kAE1 are localised to the TGN. Nonpolarised MDCKI cells stably expressing Nt $\Delta$ 1-360AE1 (a), Nt $\Delta$ 1-338AE1 (b), Nt $\Delta$ 1-292AE1 (c), Nt $\Delta$ 1-269AE1 (d) or Nt $\Delta$ 1-155AE1 (e) or transiently expressing Nt $\Delta$ 1-338AE1(Y359A) (f), Nt $\Delta$ 1-244AE1 (g) and Nt $\Delta$ 1-214AE1 (h) were fixed and immunostained with Bric170 and a suitable secondary antibody. Only the Nt $\Delta$ 1-155AE1 truncation was correctly localised to the plasma membrane, whereas the other mutants were localised to the TGN. Merged image in k shows the overlap between Nt $\Delta$ 1-338AE1 detected with Bric170 (i) and TGN38 detected with a rabbit anti-TGN38 antibody (j). Mutation of Y359 to alanine did not alleviate the TGN trafficking of the Nt $\Delta$ 1-338AE1 protein when transiently expressed in MDCKI cells (compare f with b). Therefore inclusion of even small sections of the N-terminus to the Nt $\Delta$ 1-360AE1 protein appears to disrupt the steady state localisation of kAE1. Scale bar: 30  $\mu$ m.



**Fig. 4.** Pervanadate induces kAE1 phosphorylation on residues Y359 and Y904 and is sensitive to Src kinase family inhibitors. (A,B) Western blots of Bric170 immunoprecipitates from MDCKI or MDCKI-kAE1 cells treated with or without pervanadate for the indicated times. The membranes were sequentially probed with anti-Y904-P, anti-AE1Ct and anti-Y359-P, stripping after each antibody application. (A) kAE1 phosphorylation on Y359 and Y904 is detectable by the phosphospecific antibodies within 1 minute and the majority of the protein is phosphorylated within 5 minutes. The blot shown is representative of at least eight similar experiments. (B) MDCKI-kAE1 cells were preincubated with Src kinase inhibitors 25  $\mu$ M PP1, 25  $\mu$ M PP2, 25  $\mu$ M PP3 (inactive analogue), 2  $\mu$ M staurosporine (Stauro), 100  $\mu$ M genistein (Gen), 5  $\mu$ M herbimycin (Herb) and 25  $\mu$ M SU6656 for 30 minutes before addition of 200  $\mu$ M pervanadate for 5 minutes. kAE1 phosphorylation on residues Y359 and Y904 was inhibited by both PP1 and PP2, and to a lesser extent by staurosporine and SU6656. The broad-spectrum Src kinase inhibitors genistein and herbimycin did not inhibit kAE1 phosphorylation under the conditions tested. The blot shown is representative of at least three independent experiments for each inhibitor.

Y904. Importantly, pH 8.7 medium with NaOH did not induce a similar phosphorylation of kAE1 under these conditions. Therefore, it is a high concentration of  $\text{NaHCO}_3$  rather than the high extracellular pH that induces the tyrosine phosphorylation of kAE1 in MDCKI cells. Although 514 mM  $\text{NaHCO}_3$  treatment induces kAE1 phosphorylation, there is no dramatic change in cellular tyrosine phosphorylation levels, unlike that occurring during pervanadate treatment (supplementary material Fig. S5).

We were concerned that 514 mM  $\text{NaHCO}_3$  does not reflect a physiological level of bicarbonate experienced in the kidney [typically 30 mM  $\text{NaHCO}_3$  serum levels in animal models of alkalosis (Sabolic et al., 1997)]. We therefore tested a range of bicarbonate concentrations and 300 mM was the minimum bicarbonate concentration required to induce kAE1 phosphorylation, but 514 mM still gave the most robust kAE1 phosphorylation response (see supplementary material Fig. S6A). The phosphorylation induced by  $\text{NaHCO}_3$  was inhibited by PP1 and PP2, suggesting that a Src kinase was responsible for the majority of this phosphorylation (supplementary material Fig. S6B). The inclusion of an excess of 1 mM anion exchanger inhibitor 4,4-diisothio-cyanostilbene-2,2-disulfonic acid (DIDS) with the incubation medium containing 514 mM  $\text{NaHCO}_3$  (Fig. 5B; supplementary material Fig. S6C,D), did not affect kAE1 tyrosine phosphorylation. This suggests that it is unlikely that alkalinisation

of the cell interior by kAE1  $\text{HCO}_3^-/\text{Cl}^-$  exchange causes the phosphorylation seen with  $\text{NaHCO}_3$ .

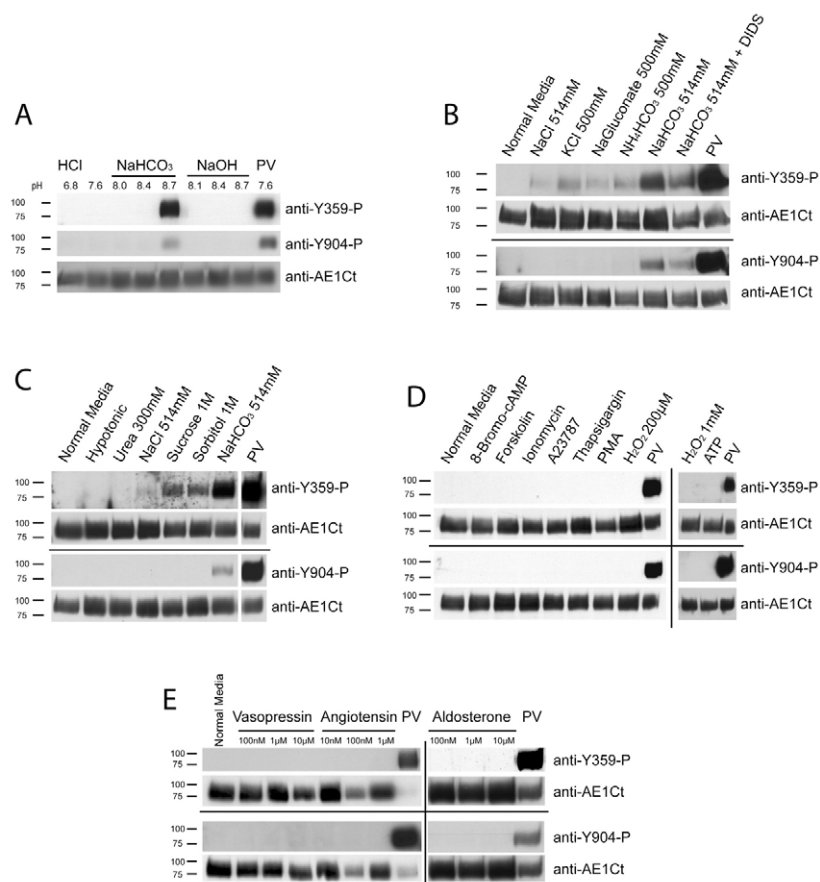
Fig. 5B shows that adding 514 mM NaCl (lane 2), 500 mM KCl (lane 3), 500 mM sodium gluconate (lane 4) or 500 mM  $\text{NH}_4\text{HCO}_3$  (lane 5) all induced similar low levels of phosphorylation of Y359 kAE1 and no detectable Y904 phosphorylation. A combination of 514 mM NaCl and pH 8.7 with NaOH did not have an additive effect on kAE1 phosphorylation (results not shown). Therefore, hypertonicity is able to induce low levels of Y359 phosphorylation of kAE1 but not to the levels observed with 514 mM  $\text{NaHCO}_3$ . Hyperosmotic stress using 1 M sucrose or 1 M sorbitol (Fig. 5C, lanes 5 and 6) causes phosphorylation of kAE1, whereas neither hypotonic medium (diluted twofold in water) (Fig. 5C, lane 2) nor the addition of 300 mM urea to induce cell swelling (Fig. 5C, lane 3) influenced kAE1 phosphorylation.

We also explored the ability of a range of other stimuli to induce kAE1 phosphorylation in MDCKI-kAE1 cells, namely, raised cAMP levels (using 8-bromo-cAMP or forskolin), increased  $\text{Ca}^{2+}$  (ionomycin, A23187 or thapsigargin) and the reagents PMA,  $\text{H}_2\text{O}_2$  and ATP (Fig. 5D). We also tested the effects of aldosterone, angiotensin II and vasopressin (dDAVP), hormones known to influence acid-base metabolism in the kidney (Wagner et al., 2004) (Fig. 5E). None of these stimuli had a detectable effect on kAE1 phosphorylation in MDCKI-kAE1 cells under the conditions tested. Taken together, these results underline the specificity of the phosphorylation response observed with high levels of  $\text{NaHCO}_3$ , hypertonicity or hyperosmolarity.

#### Tyrosine phosphorylation influences the localisation of kAE1

Since both Y359 and Y904 are critical for trafficking and are phosphorylated after pervanadate treatment or high concentrations of bicarbonate, we tested the effects of phosphorylation on kAE1 localisation. Polarised MDCKI-kAE1 cells grown on filters were treated with either 200  $\mu$ M pervanadate or 514 mM  $\text{NaHCO}_3$  and the localisation of kAE1 investigated by confocal microscopy. We found that treatment with pervanadate altered the steady state localisation of kAE1 in our model system with internalisation of kAE1 evident within 30 minutes (Fig. 6). Pervanadate treatment is known to affect tight junction integrity (Collares-Buzato et al., 1998). However, the kAE1 localisation seen here was not simply a redistribution of kAE1 due to alterations in tight junction integrity induced by pervanadate, because no kAE1 was detected at the apical surface using the extracellular FITC-Bric6, even after a 1 hour treatment with pervanadate (results not shown). In addition, we also tested the effects of pervanadate on MDCKI cells stably expressing kAE1 constructs lacking the N-terminal Y359 (kAE1Y359A and Nt  $\Delta$ 1-360AE1) or the C-terminal Y904 (kAE1R901Stop and kAE1Y904A,V907A) which are targeted apically (Toye et al., 2004). Importantly, pervanadate treatment for 30 minutes did not alter the apical localisation of these kAE1 mutant proteins (see Fig. 6E,F,G,H).

Antibody-uptake experiments on nonpolarised MDCKI-kAE1 cells, using FITC-Bric6 (Toye et al., 2004; Toye et al., 2008) were also conducted. Although some internalisation of FITC-Bric6 was detectable in control kAE1 cells (Fig. 7C,E), pervanadate treatment caused the majority of the surface-labelled kAE1 to internalise (Fig. 7D,G, as judged by resistance to acid washing see Fig. 7H). We assume that internalised kAE1 after pervanadate treatment is residing in an intermediate endocytic compartment after 30 minutes, because the majority of the internalised antibody fluorescence did not overlap with early endosomal marker EEA1, or a late endosomal



**Fig. 5.** Sodium bicarbonate and hypertonicity or hyperosmolarity induce kAE1 phosphorylation. A range of treatments were added to MDCKI-kAE1 cells. Cells were then lysed and two immunoprecipitations conducted per 10 cm<sup>2</sup> dish using Bric170. The immunoprecipitates were run on duplicate 8% SDS-PAGE gels and blots probed with either anti-Y359-P or anti-Y904-P, stripped and probed with anti-AE1Ct. A 5 minute pervanadate-positive control is included, loaded at half an immunoprecipitation equivalent to reduce the signal strength. (A) Effect of 1 hour exposure to either acidic pH 6.8 or increasing pH with either NaOH or NaHCO<sub>3</sub>. Only the pH 8.7 NaHCO<sub>3</sub> medium induced kAE1 phosphorylation (on both Y359 and Y904) in MDCKI cells. (B) Effect of 1 hour exposure to various hypertonic media on kAE1 phosphorylation. Hypertonic solutions induce phosphorylation of kAE1 on Y359 but not Y904. NaHCO<sub>3</sub> induced a consistently stronger response than hypertonicity alone and this was not inhibited by 1 mM DIDS (see supplementary material Fig. S6D for quantification). (C) Comparison of the effects of hypotonic medium (medium diluted 1:1 with water), cell swelling induced by 300 mM urea, 514 mM NaCl, 1 M sucrose or 1 M sorbitol. Hyperosmotic sucrose or sorbitol induced a greater phosphorylation of kAE1 than NaCl but this was still not as great as the effects of NaHCO<sub>3</sub>. The application of a hypotonic solution or cell swelling induced by urea had no effect on kAE1 phosphorylation. (D) Effect of incubation with 1 mM 8-bromo-cAMP, 60 μM forskolin, 5 μM ionomycin, 5 μM A23187, 1 μM thapsigargin, 10 μM PMA, either 200 μM H<sub>2</sub>O<sub>2</sub> or 1 mM H<sub>2</sub>O<sub>2</sub> and 100 μM ATP for 1 hour on MDCKI-kAE1 cells. No kAE1 phosphorylation was observed under the conditions tested using any of these reagents. Results representative of three separate experiments. (E) Effect of 1 hour exposure to vasopressin, angiotensin II or aldosterone. None of these hormones induced kAE1 phosphorylation under the conditions tested. Representative of at least two separate experiments. PV, pervanadate.

marker, such as LAMP-1, nor is it recycled back to the TGN (supplementary material Fig. S7).

When similar experiments were conducted on MDCKI-kAE1 cells exposed to high concentrations of NaHCO<sub>3</sub>, kAE1 remained at the basolateral membrane in polarised cells (Fig. 6D), and no internalisation of FITC-Bric6 was observed in nonpolarised cells (Fig. 7I). Hypertonicity is known to perturb endocytosis by disrupting the formation of clathrin lattices at the plasma membrane (Hansen et al., 1993). Consistent with this, we found that application of hypertonic NaCl or 1 M sucrose blocked pervanadate-induced

internalisation of kAE1 in nonpolarised and polarised cells (Fig. 7J,K; supplementary material Fig. S8). Thus, although high concentrations of sodium bicarbonate induce kAE1 phosphorylation, the hypertonicity of this solution prevented us from establishing the effects on trafficking in this cell culture system.

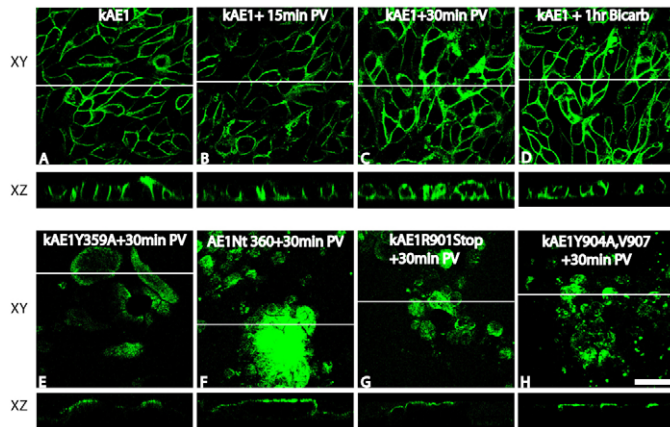
Since both kAE1Y359A and NtΔ1-360AE1 reach the plasma membrane in nonpolarised cells (see Fig. 2b and Fig. 3a), we conducted FITC-Bric6 antibody-uptake experiments on these kAE1 mutants. The majority of kAE1Y359A cells had rapidly internalised bound FITC-Bric6 within 30 minutes in our assay and the application of pervanadate did not influence the localisation of this mutant (compare Fig. 7M with 7N). Although we did not detect phosphorylation of Y904 on kAE1Y359A or NtΔ1-360AE1 under the conditions used for our immunoprecipitations (supplementary material Fig. S1D), both kAE1Y359A and NtΔ1-360AE1 internalisation was inhibited by incubation with 25 μM PP2 (see Fig. 7P,R) but not the inactive analogue PP3 (see Fig. 7O,Q), which is consistent with the suggestion that tyrosine phosphorylation influences kAE1 localisation. Importantly, application of PP2 did not alter normal kAE1 internalisation (compare Fig. 7S with 7T), suggesting that phosphorylation is not responsible for internalisation of kAE1 under normal culture conditions or that normal kAE1 internalises via another pathway in the presence of Src kinase inhibitor PP2.

## Discussion

### Y359 is a kAE1 N-terminal trafficking determinant

Previous work demonstrated that kAE1 basolateral trafficking requires at least two targeting determinants, one in the cytosolic N-terminus and Y359 in the cytosolic C-terminus (Devonald et al., 2003; Toye et al., 2004). This work has identified the N-terminal determinant as Y359, because mutagenesis of this single residue to alanine results in apical mistargeting, a localisation consistent with the loss of a basolateral determinant. An alignment of AE1 proteins from several species (supplementary material Fig. S2) highlights that Y359 is conserved in orthologues from human, dog, horse and mouse, but is absent from chicken, cows and

sheep. The C-terminal Y904 residue is conserved across all species sequences analysed (supplementary material Fig. S2). Chicken kAE1 has two tyrosine-based determinants in the N-terminus essential for basolateral localisation (Adair-Kirk et al., 1999; Adair-Kirk et al., 2003), but these are absent in human kAE1. It is therefore possible that cows and sheep lost their requirement for the Y359 determinant owing to their production of alkaline urine, or they may have evolved alternative residues for kAE1 trafficking, like chicken kAE1. Furthermore, we note that a tyrosine residue equivalent to Y904 in the C-terminus is a conserved feature in other



**Fig. 6.** Pervanadate-induced tyrosine phosphorylation influences basolateral kAE1 localisation. MDCKI-cells stably expressing kAE1 (A,B,C,D) or four apically targeted kAE1 mutants kAE1Y359A (E), NtΔ1-360AE1 (F), kAE1(Y904A,Y907A) (H) or kAE1R901Stop (G) polarised on filters. 200  $\mu$ M pervanadate (PV) was added to the cells in B-C, E-H or 514 mM NaHCO<sub>3</sub> added to D for the times indicated. kAE1 was detected using AE1 monoclonal antibody Bric170 and a suitable secondary antibody. The upper micrographs are focal planes taken parallel to the epithelium (X-Y) near the centre for kAE1 or at the apical membrane for the kAE1 mutants. The lower micrographs show focal planes perpendicular to the epithelium (X-Z) along the white line in the X-Y image. Normal kAE1 is at the basolateral membrane in untreated control cells (A) and those treated with pervanadate for 15 minutes (B). kAE1 localisation was intracellular after 30 minutes of pervanadate treatment in C. (D) NaHCO<sub>3</sub> had no effect on kAE1 localisation. (E-H) Pervanadate treatment for 30 minutes did not alter the apical localisation of kAE1 mutants that lack either Y359 or Y904. Scale bar: 30  $\mu$ m.

anion exchanger family members (AE2 and AE3) but Y359 is not conserved. Thus, Y359 has a role specific to kAE1 basolateral trafficking in the  $\alpha$ -IC of humans and animals which usually generate acidic urine.

The sequence of events and cellular machinery whereby Y359 and Y904 residues co-ordinate kAE1 basolateral localisation has yet to be determined. Both Y359 and Y904 appear to be equally important for kAE1 polarised trafficking, because the removal of either tyrosine residue results in apical mistargeting. It is therefore possible that Y359 recruits factors essential for basolateral sorting or retention. The sequence Y359xxL conforms to the consensus motif Yxx $\Phi$ , which may bind to medium chain subunits of adaptor complexes involved in clathrin dependent and independent trafficking (Aguilar et al., 2001; Boehm and Bonifacio, 2001; Dell'Angelica et al., 1997; Ohno et al., 1996; Stephens and Banting, 1998). However, adaptor complex AP-1B, responsible for some basolateral protein trafficking (Gan et al., 2002) is not required for kAE1 sorting to the basolateral membrane (Toye et al., 2004).

In erythrocytes, the Y359-P residue recruits the tyrosine phosphatase SHP-2 (PTPN11) and the bound SHP-2 dephosphorylates Y904 (Bordin et al., 2002). If SHP-2 also associates with kAE1 via Y359 in ICs, this interaction may be important for retention or recycling of kAE1 at the basolateral membrane (see Fig. 8) and would explain a requirement for both Y359 and Y904 in kAE1 basolateral localisation (Toye, 2005). Our observation that kAE1 rapidly internalises in the absence of a Y359 residue in kAE1Y359A- and NtΔ1-360AE1-expressing cells, and that this process is inhibited by Src kinase inhibitor PP2 (see Fig. 7), is consistent with this model. However, we can only indirectly

implicate Y904 phosphorylation as being responsible for the rapid internalisation of these kAE1 mutants, because no phosphorylated Y904 was detectable using our phosphospecific antibodies in kAE1 immunoprecipitations from Y359A- and NtΔ1-360AE1-expressing cells. This may be explained by phosphorylation of Y904 being a transient event or because phosphorylation on Y904 is unstable under the conditions of cell lysis, as previously experienced with proteins where tyrosine phosphorylation is implicated in trafficking (e.g. Anderson et al., 2005). Further work is underway to determine whether the SHP-2 or another phosphatase has a role in kAE1 basolateral trafficking.

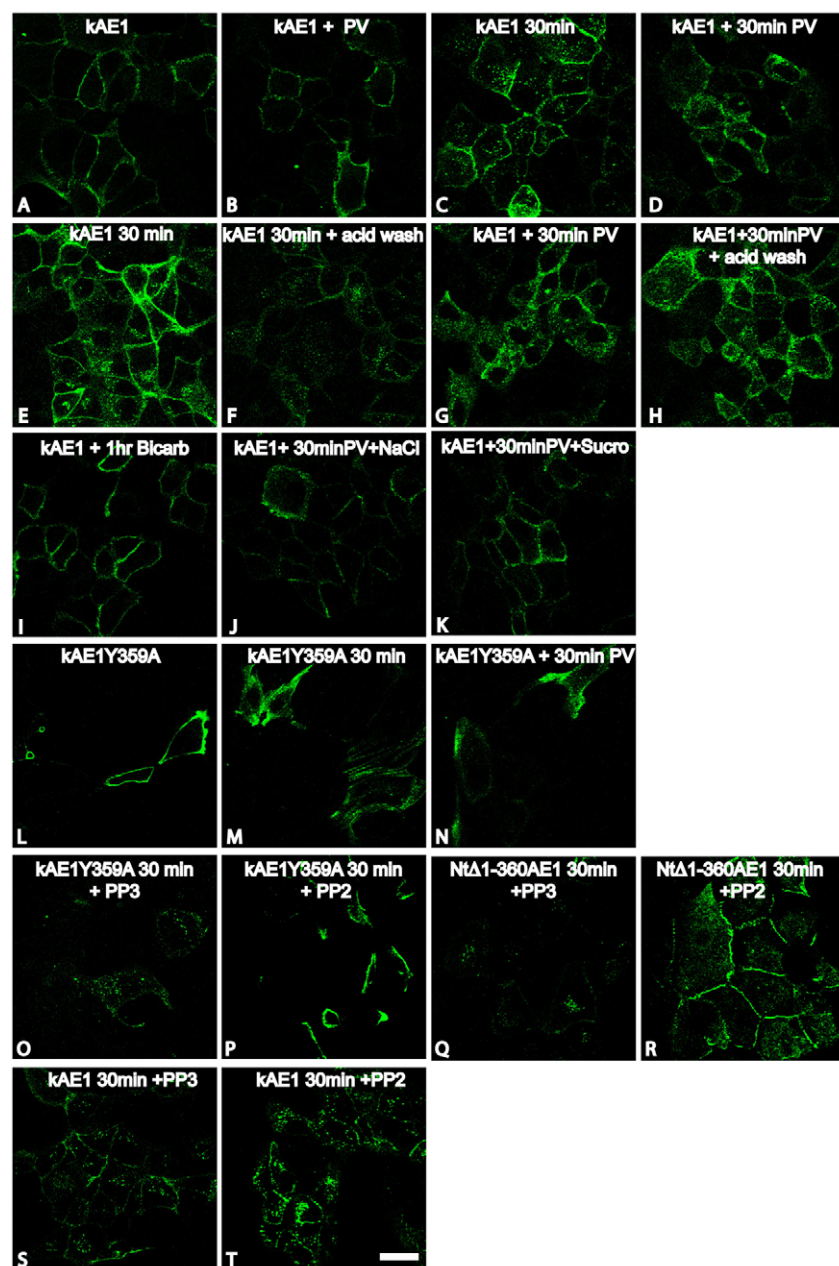
It is surprising that despite the presence of Y359, the majority of N-terminal truncations of kAE1 tested had a TGN localisation at steady state. Therefore, we cannot exclude the possibility that additional regions of the N-terminus may contribute to the correct trafficking of kAE1. It is possible that the distal N-terminal portion of kAE1 requires proximal sequences for correct folding or presentation of trafficking motifs, but this seems unlikely because the largest N-terminal truncation tested (NtΔ1-360AE1) reaches the plasma membrane. Alternatively, a cryptic TGN localisation signal or aberrant protein interaction might occur with the N-terminus, which becomes inactivated upon formation of a more native N-terminal structure. However, we feel that a more likely explanation is that the inclusion of incorrectly folded N-terminal sequences disrupts kAE1 localisation by increasing kAE1 aggregation and thus affecting its incorporation into transport vesicles from the TGN.

Recently, kAE1 has been demonstrated to interact with the serine/threonine kinase and actin-binding protein integrin-linked kinase (ILK) (Keskanokwong et al., 2007). ILK associates with kAE1 via a calponin-homology (CH) domain on kAE1 (residues 27-189). The NtΔ1-155AE1 construct removes the majority of the CH domain (N-terminal residues 36-98 show the greatest similarity to CH domain of spectrin) but is correctly targeted in nonpolarised cells and polarised cells. Thus, either residues 155-189 comprise the ILK-binding site or an interaction with ILK is not essential for kAE1 trafficking in kidney cells.

#### Phosphorylation of kAE1

We have shown that no phosphorylation was detectable on either Y359 or Y904 in MDCKI-kAE1 cells under normal culture conditions, but both Y359 and Y904 residues were phosphorylated in the presence of the tyrosine phosphatase inhibitor pervanadate. As far as we are aware, this is the first demonstration that kAE1 is phosphorylated on Y359 and Y904 in kidney cells. Hydrogen peroxide alone can inhibit protein tyrosine phosphatases by oxidising the catalytic cysteine residues of phosphatases (Hecht, 1992), but even 1 mM H<sub>2</sub>O<sub>2</sub> exposure had no effect on kAE1 phosphorylation and H<sub>2</sub>O<sub>2</sub> did not induce phosphorylation of eAE1 (Harrison et al., 1994). Of interest, the phosphatase SHP-2 (which interacts with eAE1 at Y359 and therefore possibly kAE1) can associate with catalase, providing protection from H<sub>2</sub>O<sub>2</sub> oxidation (Yano et al., 2004).

The kinase that phosphorylates kAE1 on Y359 and Y904 in MDCKI cells has a profile similar to the kinase responsible for AE1 phosphorylation in erythrocytes, which is also sensitive to Src kinase inhibitors PP1 and PP2. In erythrocytes, AE1 is sequentially phosphorylated by Syk and Lyn kinase and pre-treatment of eAE1 with Syk kinase (which phosphorylates eAE1 on residues Y8, Y27) is required for efficient phosphorylation by Lyn kinase on Y359 and Y904 (Brunati et al., 2000). Band 3 Neapolis, which lacks the first N-terminal 11 amino acids of eAE1 (including Y8), is not



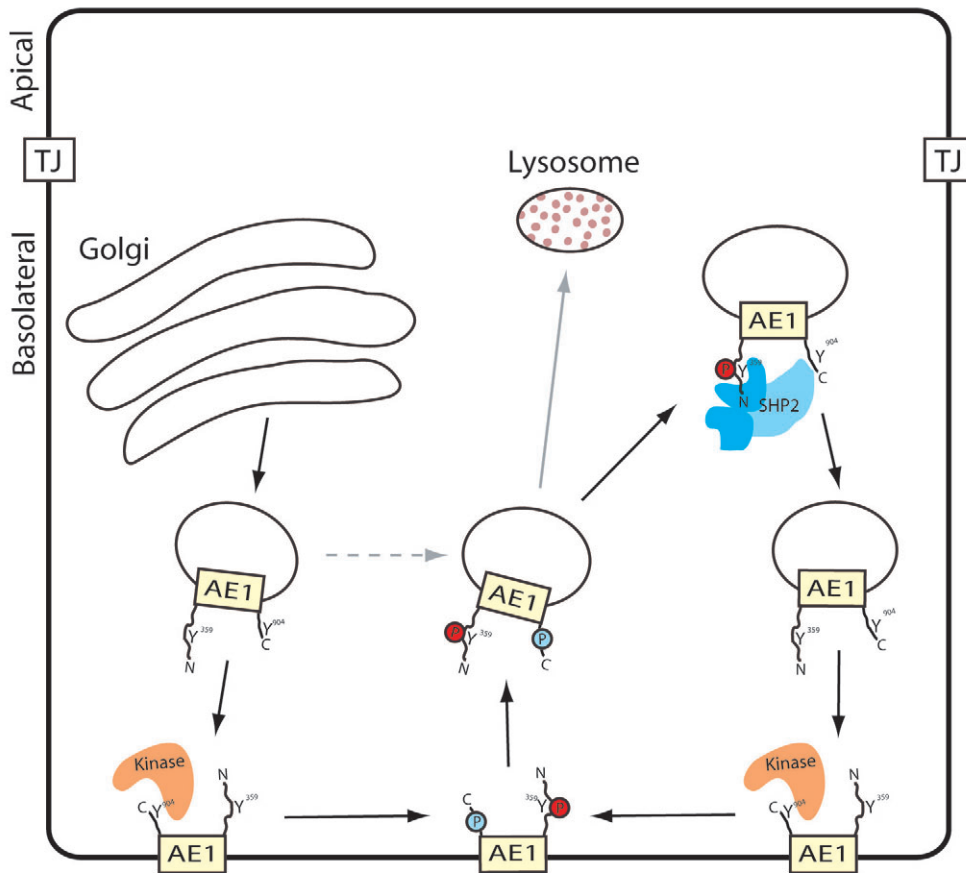
**Fig. 7.** Antibody uptake assay shows that phosphorylated kAE1 rapidly internalises from the plasma membrane in nonpolarised cells. MDCKI-kAE1, MDCKI-kAE1Y359A or MDCKI-NtΔ1-360AE1 cells were seeded onto coverslips and an antibody-uptake assay using FITC-Bric6 conducted under various conditions. (A-H) Pervanadate induces kAE1 internalisation. The majority of the kAE1 FITC-Bric6 is at the cell surface before internalisation (A,B). (C,D) FITC-Bric6 internalisation at 37°C for 30 minutes in the absence and presence of pervanadate (PV). (E-H) FITC-Bric6 is susceptible to acid wash in control cells (compare E and F) but pervanadate-treated cells are resistant to this process (compare G and H), consistent with the majority of the kAE1 having been internalised. (I-K) Hypertonicity inhibits kAE1 internalisation. (I) There is a lack of FITC-Bric6 internalisation following 1 hour exposure to 514 mM NaHCO<sub>3</sub>. (J,K) In the presence of 514 mM NaCl or 1 M sucrose, pervanadate-induced FITC-Bric6 internalisation is inhibited. (L-T) kAE1 mutants that lack Y359 have a rapid internalisation of FITC-Bric6 and this is inhibited by Src kinase inhibitor PP2. (L) FITC-Bric6 binding to kAE1Y359A cells before internalisation is allowed to commence. (M,N) kAE1Y359A uptake in the absence or presence of pervanadate. This demonstrates that Y359A rapidly internalises and there is no obvious difference between antibody uptake in the presence or absence of pervanadate. (O-T) Comparison of kAE1Y359A (O and P), Nt1-360AE1 (Q and R) or kAE1 (S and T) treated with either PP3 or PP2 as indicated. Scale bar: 30 μm.

phosphorylated in pervanadate-treated erythrocytes (Perrotta et al., 2005). Since kAE1 is truncated by an additional 54 amino acids compared with Band 3 Neapolis and is phosphorylated during pervanadate treatment, either kAE1 phosphorylation is different in the kidney because of altered accessibility to these phosphorylation sites by Lyn kinase, because of structural differences in the N termini between kAE1 and eAE1, or because an alternative kinase is responsible for kAE1 phosphorylation in kidney cells.

In addition to the results seen with pervanadate (Harrison et al., 1994), human eAE1 is phosphorylated in cells exposed to hypertonic medium (Minetti et al., 1996; Minetti et al., 1998) or calcium (Zipser et al., 2002). We tested a range of potential stimuli on MDCKI-kAE1 cells for their effects on kAE1 phosphorylation. The phosphorylation of kAE1 was specific to high nonphysiological concentrations of sodium bicarbonate, hypertonicity or hyperosmolarity (see Fig. 5). A wide range of stimuli did not induce

phosphorylation of these kAE1 tyrosine residues in this culture system (see Fig. 5). The lack of an effect of increasing calcium levels suggests that some aspects of tyrosine phosphorylation differ between eAE1 and kAE1, possibly because of an inability of the truncated kAE1 to interact with the phosphatase PTP-1B responsible for the calcium sensitivity of eAE1 (Zipser et al., 2002).

Hypertonicity is known to induce tyrosine phosphorylation of AE1 in human red blood cells (Minetti et al., 1996; Minetti et al., 1998) and hypertonicity activates tyrosine kinases by cell shrinkage (Kapus et al., 1999; Krump et al., 1997). We do not know why only Y359 was phosphorylated during hypertonic or hyperosmotic stress. It is possible that the association of SHP-2 or another phosphatase with this phosphorylated residue enables the dephosphorylation of Y904, as is proposed to occur in erythrocytes (Bordin et al., 2002). We assume that the rise in intracellular pH that accompanies hypertonic stress is not the trigger for kAE1



**Fig. 8.** Scheme to explain the role of Y359 and Y904 in regulating kAE1 localisation. kAE1 has two tyrosine residues, one in the N-terminus and one in the C-terminus, that are critical for basolateral localisation. We have shown that both of these tyrosines Y359 and Y904 can be phosphorylated. We propose that when Y359 and Y904 are phosphorylated at the plasma membrane, this marks the protein for internalisation by endocytosis. If Y359 is phosphorylated we hypothesise that this recruits a phosphatase (in this case SHP-2 via SH2 domain interaction), which then dephosphorylates Y904, blocking phosphorylation specific internalisation or revealing the basolateral targeting motif for recycling kAE1 back to the plasma membrane.

phosphorylation, because our preliminary experiments showed that treatment of MDCKI-kAE1 cells for 10, 15 or 30 minutes with 40 mM  $\text{NH}_4\text{Cl}$  (a reagent that initially induces a rise in intracellular pH) did not induce phosphorylation (results not shown).

The cellular mechanism behind the activation of phosphorylation of Y359 and Y904 after exposure to high bicarbonate is currently unknown. We originally favoured the possibility that the cells are responding to extracellular bicarbonate rather than the hypertonicity of the solution, because  $\text{NaHCO}_3$  induced the most robust level of phosphorylation of all the solutes tested, and the presence of the anion exchanger inhibitor DIDS did not inhibit this response. However, the high concentrations of extracellular  $\text{NaHCO}_3$  necessary to observe phosphorylation in our model system do not reflect a physiological level of bicarbonate experienced in the kidney.

Cells increase their volume after shrinkage by the compensatory net inward movement of ions, for example, by coupling the activity of  $\text{Na}^+/\text{H}^+$  exchange (e.g. NHE1) with  $\text{HCO}_3^-/\text{Cl}^-$  exchangers (O'Neill, 1999). This results in a net influx of  $\text{Na}^+$  and  $\text{Cl}^-$ , and efflux of  $\text{H}^+$  and  $\text{HCO}_3^-$ . The extracellular  $\text{H}^+$  and  $\text{HCO}_3^-$  ions combine to generate  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , which regenerate  $\text{H}^+$  and  $\text{HCO}_3^-$  ions upon diffusion of  $\text{CO}_2$  into the cell. It is conceivable that under hypertonic conditions, a high extracellular bicarbonate concentration interferes with the compensatory anion exchanger activity, perhaps by allowing futile  $\text{HCO}_3^-/\text{HCO}_3^-$  exchange. In support of this, preliminary experiments have shown that exposure to 514 mM NaCl in the presence of DIDS induces phosphorylation of kAE1 to a level similar to that observed with 514 mM sodium bicarbonate (results not shown). We do not know why 500 mM  $\text{NH}_4\text{HCO}_3$  did not have

a similar effect as  $\text{NaHCO}_3$  but we assume the alkalinity of this solution (pH 8.6) ensures that  $\text{NH}_3$  formation is favoured, with the  $\text{H}^+$  donated to  $\text{HCO}_3^-$  forming  $\text{H}_2\text{O}$  and  $\text{CO}_2$ . It is possible that the  $\text{CO}_2$  generated by this medium and consequential  $\text{H}^+$  and  $\text{HCO}_3^-$  regeneration inside the cell, circumvents any disruption of anion exchanger activity caused by high concentrations of extracellular bicarbonate.

Since the mechanisms underlying kAE1 phosphorylation are currently unknown and our transfected cell model system may not reflect all the features of an  $\alpha$ -IC in the kidney, it is possible that some stimuli tested might still have an important role in influencing kAE1 levels at the basolateral membrane in ICs. Both aldosterone and angiotensin II treatment increase intracellular  $\text{Ca}^{2+}$  levels (Gekle et al., 2002; Oliveira-Souza and De Mello-Aires, 2000) in MDCK cells, whereas vasopressin can increase cAMP levels or  $\text{Ca}^{2+}$  by V1 and V2 receptors, respectively (Oliveira-Souza et al., 2004). All three hormones appear to increase proton transport and bicarbonate reabsorption (Wagner et al., 2004) implicating  $\text{Ca}^{2+}$  and cAMP as potential signals for acidosis. Therefore, it is interesting that increasing intracellular  $\text{Ca}^{2+}$  or cAMP levels did not induce kAE1 phosphorylation in our studies.

#### Tyrosine phosphorylation and its potential role in kAE1 trafficking in health and disease

Our data suggest that phosphorylation of both Y359 and Y904 triggers the internalisation of kAE1 from the plasma membrane in nonpolarised cells or the basolateral membrane in polarised cells. Interestingly, no internalisation was observed for apically targeted kAE1 constructs lacking either Y359 or Y904 in the

presence of pervanadate. However, in nonpolarised cells, the absence of Y359 causes rapid internalisation of kAE1 in a process that is Src kinase dependant. This observation suggests that apical internalisation of kAE1 might utilise different machinery or has different trafficking kinetics to kAE1 internalisation from the basolateral membrane. This result is similar to observations of CEACAM1-L trafficking in MDCK cells, where pervanadate treatment caused rapid internalisation of basolateral localised CEACAM1-L, but left apical targeted protein unaffected (Sundberg et al., 2004).

Given the knowledge that both Y359 and Y904 are critical for targeting to the basolateral membrane and tyrosine phosphorylation alters the localisation of kAE1, we can extrapolate from these results to hypothesise that ICs might regulate the levels of kAE1 at plasma membrane during chronic acid-base changes by altering the activity of tyrosine kinases or phosphatases. This provides an attractive mechanism that would help explain observations from animal studies, that suggest that ICs increase kAE1 levels at the basolateral membrane during chronic metabolic acidosis and reduce kAE1 levels during acute metabolic alkalosis (Fejes-Toth et al., 1994; Huber et al., 1999; Sabolic et al., 1997; Verlander et al., 1994). This is similar to the regulation of Kir1.1 (ROMK) levels by tyrosine phosphorylation, depending on the availability of dietary potassium (Wang et al., 2002; Wei et al., 2001). The observation here that hypertonicity also influences kAE1 phosphorylation is consistent with this hypothesis, because hypertonicity reduced kAE1 levels in the outer medulla in mice (Barone et al., 2004).

In addition to a putative role in the regulation of kAE1 localisation during systemic acid-base changes, it is also conceivable that inappropriate phosphorylation of kAE1 could result in dRTA. This is because structural changes induced by certain AE1 gene mutations may result in altered accessibility to kinases or phosphatases, thus affecting the basolateral localisation of kAE1. In support of this suggestion, we are beginning to elucidate a role for aberrant phosphorylation in the dominant dRTA mutation A858D (Bruce et al., 2000), which is correctly targeted to the basolateral membrane (Cordat and Reithmeier, 2006) but is rapidly endocytosed (A.M.T., unpublished observations).

In summary, we have identified a critical tyrosine, Y359, in the N-terminus of kAE1 that is essential alongside a C-terminal Y904 residue for correct basolateral trafficking. We generated and characterised novel tools for studying AE1 tyrosine phosphorylation, demonstrated that kAE1 is phosphorylated under specific conditions and that tyrosine phosphorylation can influence kAE1 localisation. This has allowed us to propose a mechanism whereby phosphorylation of kAE1 regulates its localisation during acid-base changes. Further studies are necessary to investigate this intriguing proposition further, to determine whether kAE1 is phosphorylated in animal models subjected to chronic alkalosis, whether there is aberrant phosphorylation of kAE1 in other known dRTA mutations and to identify the cellular machinery required for correct kAE1 trafficking and regulation.

## Materials and Methods

### Antibodies

Monoclonal antibodies used were: Bric170, which recognises an epitope at the N-termini of kAE1 (Toye et al., 2004) and FITC-Bric6, which reacts with an extracellular kAE1 epitope (Smythe et al., 1995). Rabbit anti-calnexin was from Stressgen (San Diego, CA), rabbit anti-furin from Affinity Bioreagents (Golden, CO), rabbit anti-LAMP-1 (270c) was raised against the peptide sequence KRSHAGYQTI, and rabbit anti- $\beta$ -catenin was from abCam (Cambridge, UK). The rabbit anti-TGN38 (Wilde et al., 1992) and rabbit EEA1 was kindly provided by George Banting (University of Bristol, UK). The rabbit C-terminal AE1 antibody

was raised against a peptide corresponding to residues 881-911 (Toye et al., 2008). Secondary antibodies were goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 594 (Molecular Probes, Invitrogen, Paisley, UK). In some instances a biotin-conjugated anti-mouse antibody and FITC-labelled anti-biotin antibody (Vector Labs, Burlingame, CA) were used.

The phosphospecific antibodies to Y359-P and Y904-P were raised against the synthetic peptides SSFYPhosKGLDLGC and GRDEYPhosDEVAGC, respectively (where the GC acts as a spacer and C-terminal coupling site). All peptides were synthesised to a purity of >95% by the University of Bristol Peptide Synthesis Facility, coupled to KLH and then used to immunise two rabbits for each immunogen.

### Construction of kAE1 mutant cDNA

The N-terminal tyrosine substitution construct kAE1 (Y359A) was made using Quikchange (Stratagene, La Jolla, CA) and pcDNA3-kAE1 as a template as instructed by the manufacturer. The N-terminal truncation constructs were made by PCR using suitable primers that incorporate an initiating methionine, using Expand Taq Polymerase (Roche Diagnostics, West Sussex, UK) and pcDNA3-kAE1 as a template as previously described (Toye et al., 2002). Constructs were TOPO-TA cloned into pcDNA3 as directed by the manufacturer (Invitrogen). All constructs were confirmed by DNA sequencing (Geneservice, University of Oxford, UK).

### MDCK cell culture and cell transfection

MDCKI cells stably expressing kAE1, NtA1-360AE1 (AE1mem), kAE1Y904F, kAE1(Y904A, Y907A) and kAE1R901Stop were already available (Toye et al., 2004). Stably transfected MDCKI cells cell lines were obtained as described previously (Toye et al., 2002). Cells were polarised by seeding on to permeable filters (Nunc, Fisher Scientific, Leicestershire, UK) as described previously (Toye et al., 2004).

### Immunofluorescence microscopy

Cells were processed for confocal microscopy as described previously (Toye et al., 2004). For antibody double labelling, cells were incubated in neat anti-AE1 Bric170 (19  $\mu$ g/ml), then anti-TGN38 (or another intracellular marker) was applied in PBS containing 4% BSA, followed by compatible goat anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 secondary antibodies. For optimal images of polarised kAE1 expression, cells were treated with 5 mM sodium butyrate for approximately 16 hours prior to fixation. In some instances, optimal imaging of MDCKI-kAE1Y359A cells grown on filters was achieved by utilising a biotin-conjugated anti-mouse antibody and FITC-labelled anti-biotin antibody (Vector labs) but this was not necessary for wild-type kAE1 or the other kAE1 mutants used. Fluorescence imaging was done using a Leica TCS-NT confocal laser-scanning microscope (Leica-Microsystems, Milton Keynes, UK) equipped with a Kr/Ar laser. For dual-labelling confocal microscopy studies, Alexa Fluor 488, FITC or Alexa Fluor 594 images were taken sequentially with excitation/emission filters set at short pass 510 nm/band pass 530 nm for Alexa Fluor 488/FITC and band pass 568 nm/long pass 590 nm for Alexa Fluor 594. Images were processed using Adobe Photoshop CS2 (version 9.0) and Adobe Illustrator CS2 (version 12.0).

### Labelling of cell-surface AE1 with extracellular anti-AE1 antibody FITC-Bric6

For cells polarised on permeable filters, intact cell layers were washed with ice-cold PBS and incubated at 4°C for 20 minutes. FITC-Bric6 in 4% BSA PBS (pre cooled to 4°C) was applied to both apical and basolateral surfaces and the cells incubated at room temperature for 1 hour, washed, fixed and imaged as described previously (Toye et al., 2004). To monitor internalisation of kAE1 in nonpolarised MDCKI cells on coverslips, cells were washed four times in ice-cold PBS buffer and chilled on ice for 10 minutes. Pre-cooled FITC-Bric6 in PBS buffer containing 4% BSA was added to the cells for 30 minutes on ice. The cells were then washed three times in PBS, prewarmed 37°C medium added immediately and antibody uptake continued for 30 minutes. To remove surface-bound antibody, coverslips were acid washed with ice-cold glycine (pH 2, 150 mM). To observe the effects of pervanadate on kAE1 internalisation, after the 30 minute application of FITC-Bric6 the cells were washed, then treated with ice-cold PBS containing 200  $\mu$ M pervanadate for 5 minutes on ice (control cells had PBS with no pervanadate) and 200  $\mu$ M pervanadate was present in the prewarmed medium step. To observe the effects of Src kinase inhibitor on kAE1 internalisation, cells were pre-incubated with 25  $\mu$ M PP2 or PP3 in ice cold PBS for 10 minutes prior to adding FITC-Bric6, and 25  $\mu$ M PP2 or PP3 were also included in the FITC-Bric6 and prewarmed medium steps. Coverslips were either fixed in 100% methanol (for immediate imaging), paraformaldehyde-saponin (for Lamp-1 staining) (Toye et al., 2004) or methanol/acetone (60/40 v/v) for colocalisation with intracellular markers, then processed for immunofluorescence.

### Cell treatment

Confluent MDCKI or MDCKI-kAE1 cells grown on 10 cm<sup>2</sup> dishes were incubated at 37°C with 5 mM sodium butyrate in DMEM medium containing 25 mM HEPES (Gibco, Invitrogen) for approximately 16 hours to induce protein expression. 10 ml fresh medium (no sodium butyrate) was then added containing the following treatments.

### Pervanadate and Src kinase inhibitors

To induce phosphorylation cells were treated with 200  $\mu$ M pervanadate, for 0, 5, 15 and 30 minutes or as indicated in the figure legend. Tyrosine kinase inhibitors PP1, PP2, PP3 (an inactive analogue) and 25  $\mu$ M SU6656, 2  $\mu$ M staurosporine, 5–10  $\mu$ M herbimycin, 100  $\mu$ M–200  $\mu$ M genistein (Calbiochem, Nottingham, UK) were added. All the inhibitors were dissolved in DMSO and preincubated with the cells for 30 minutes prior to pervanadate treatment for 5 minutes.

### Extracellular pH

The pH of DMEM media was altered by addition of HCl to pH 6.8, NaOH to pH 8.1, 8.4, and 8.7, and NaHCO<sub>3</sub> to pH 8.0, 8.4 and 8.7. Media solutions were equilibrated at 37°C, 5% CO<sub>2</sub> for 1 hour and pH confirmed before adding to cells. Growth medium was replaced by the different pH media, and then plates were incubated at 37°C, 5% CO<sub>2</sub> for 1 hour. The pH of the medium was checked after each experiment to ensure that the pH was stable for the duration of the experiment. For the treatment of cells with anion exchanger inhibitor 4,4-diisothio-cyanostilbene-2,2-disulfonic acid (DIDS), cells were pre-incubated with 1 mM DIDS (Sigma-Aldrich, Dorset, UK) in medium for 20 minutes and then 1 mM DIDS added in the 514 mM NaHCO<sub>3</sub> medium for 1 hour.

### Hypertonicity and hypotonicity

To observe the effects of hypertonic medium, 514 mM NaHCO<sub>3</sub> (BDH, Dorset UK), 514 mM NaCl (Fisher Scientific), 1 M sucrose (BDH), 1 M sorbitol (Sigma-Aldrich), 500 mM NH<sub>4</sub>HCO<sub>3</sub> (Sigma-Aldrich), 500 mM sodium gluconate (Sigma-Aldrich) or 500 mM KCl (BDH) were dissolved in fresh DMEM medium. Hypotonic medium was made by diluting medium 1:1 with water, and we induced cell swelling by adding 300 mM urea (Amresco). The different media were prepared and allowed to equilibrate for 1 hour at 37°C, 5% CO<sub>2</sub>. The medium on each plate was replaced with one of the media solutions above, and then plates were incubated at 37°C, 5% CO<sub>2</sub> for 1 hour.

### Chemical treatments

The following stimuli were added to the medium for 1 hour: 60  $\mu$ M forskolin or 1 mM thapsigargin (to raise cAMP), 5  $\mu$ M ionomycin or A23187 or 1  $\mu$ M thapsigargin (to raise intracellular calcium levels), 10  $\mu$ M phorbol 12-myristate 13-acetate (to activate PKC), 100  $\mu$ M ATP and 200  $\mu$ M or 1 mM H<sub>2</sub>O<sub>2</sub>. All chemicals were purchased from Tocris Bioscience (Bristol, UK) or Sigma-Aldrich.

### Hormones

Aldosterone (100 nM, 1  $\mu$ M, 10  $\mu$ M), angiotensin II (10 nM, 100 nM, 1  $\mu$ M) and [deamino-Cys<sup>1</sup>, D-Arg<sup>8</sup>]-vasopressin (100 nM, 1  $\mu$ M, 10  $\mu$ M) were added to the growth medium for 1 hour. All hormones were purchased from Sigma-Aldrich.

### Immunoprecipitations

Cells were quickly washed in PBS and lysed in 250  $\mu$ l 2% SDS immunoprecipitation buffer (IP buffer) [150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 10 mM sodium orthovanadate, 2 mM phenylmethanesulfonylfluoride (PMSF), Calbiochem Protease Inhibitor Cocktail set V, 1% Sigma-Aldrich Phosphatase Inhibitor Cocktail 2]. 2.5 ml IP Buffer containing 1% Triton X-100, 1% sodium deoxycholate was added to the plate. 1% BSA in immunoprecipitation buffer was then added, with 30  $\mu$ l protein-G-agarose beads (Pierce, Rockford, IL) and precleared at 4°C for 1 hour on a rotator. The lysates were transferred to Protein-G-agarose beads preloaded with anti-AE1 Bric170. After overnight incubation, beads were washed with IP buffer four times. The immune complexes were eluted from the beads with sample buffer, and then separated by 8% SDS-PAGE gels. Proteins were electroblotted on to a PVDF membrane and probed with the phosphospecific antibodies anti-Y359-P or anti-Y904-P or anti-AE1Ct. The labelled proteins were detected using a swine anti-rabbit HRP secondary antibody (Dako) and detected using Western Lightning chemiluminescence reagent (PerkinElmer, Waltham, MA) and film.

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