The Carbonyl Group of Glutamic Acid-795 Is Essential for Gastric H^+,K^+-ATPase Activity†

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ABSTRACT: To study the role of Glu^{795}, present in the fifth transmembrane domain of the α-subunit of gastric H^+,K^+-ATPase, several mutants were generated and expressed in Sf9 insect cells. The E795Q mutant had rather similar properties as the wild-type enzyme. The apparent affinity for K^+ in both the ATPase reaction and the dephosphorylation of the phosphorylated intermediate was even slightly enhanced. This indicates that the carbonyl group of Glu^{795} is sufficient for enzymatic activity. This carbonyl group, however, has to be at a particular position with respect to the other liganding groups, since the E795D and E795N mutants showed a strongly reduced ATPase activity, a lowered apparent K^+ affinity, and a decreased steady-state phosphorylation level. In the absence of a carbonyl residue at position 795, the K^+ sensitivity was either strongly decreased (E795A) or completely absent (E795L). The mutant E795L, however, showed a SCH 28080 sensitive ATPase activity in the absence of K^+, as well as an enhanced spontaneous dephosphorylation rate, that could not be further enhanced by K^+, suggesting that this mutant mimicks the filled K^+ binding pocket. The results indicate that the Glu^{795} residue is involved in K^+-stimulated ATPase activity and K^+-induced dephosphorylation of the phosphorylated intermediate. Glu^{795} might also be involved in H^+ binding during the phosphorylation step, since the mutants E795N, E795D, and E795A showed a decrease in the phosphorylation rate as well as in the apparent ATP affinity in the phosphorylation reaction. This indicates that Glu^{795} is not only involved in K^+ but might also play a role in H^+ binding.

Transport of H^+ and K^+ cations across the membrane is the main physiological function of gastric H^+,K^+-ATPase and results in an acidification of the gastric lumen. The energy needed for this transport is supplied by the hydrolysis of ATP. During the catalytic cycle, an aspartate residue present in a conserved region of the large intracellular loop between the fourth and fifth transmembrane domains of the (catalytic) α-subunit becomes phosphorylated by ATP (*1*). This is one of the main characteristics of the P_2-type ATPases, a subfamily which also includes Na^+,K^+-ATPase and Ca^{2+}-ATPases (*2*).

Up to now, one of the unresolved issues concerning these ATPases is the coupling between ATP hydrolysis and ion transport. In purified renal Na^+,K^+-ATPase, proteolytic digestion has shown that the cation occlusion cavity is within the transmembrane domains (*3*). Additionally, chemical labeling has revealed a possible role of carboxylic acids, located in the membrane-spanning regions, in cation binding (*4*−*6*). In particular, highly conserved polar residues (like aspartate and glutamate) present in transmembrane domains 4, 5, and 6 play a pivotal role with respect to cation binding in Na^+,K^+-ATPase (*7*−*13*), SR[1] Ca^{2+}-ATPase (*12*−*17*), and gastric H^+,K^+-ATPase (*18*−*22*). The putative transmembrane regions M5 and M6 of the gastric H^+,K^+-ATPase contain three of these negatively charged residues (Glu^{795}, Glu^{820}, and Asp^{824}). Mutagenesis of Asp^{824} to an Asn resulted in an enzyme which could no longer be phosphorylated (*21*). Several Glu^{820} mutants, on the other hand, could be phosphorylated but, with the exception of the E820D and E820A mutants, showed no K^+-stimulated dephosphorylation and no K^+-stimulated ATPase activity (*18*). Some of these mutants even demonstrated an enhanced spontaneous dephosphorylation rate and a K^+-independent (constitutive) ATPase activity (*19*). This led to the hypothesis that Glu^{820} is part of the K^+-binding pocket and that K^+ ions neutralize the negatively charged binding pocket, which normally inhibits the dephosphorylation process (*19*).

Concerning the third negatively charged residue in this M5 and M6 region, we previously found no striking differences between the properties of the E795Q mutant and the wild-type enzyme, with respect to K^+-stimulated ATPase activity and K^+-induced dephosphorylation (*21*). This apparently contrasts with Na^+,K^+-ATPase and SR type Ca^{2+}-ATPase where the analogous residues are thought to play an essential role in cation binding and transport (*14*, *23*−*25*).

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[1] Abbreviations: SR, sarcoplasmic reticulum; E-P, phosphorylated intermediate; SCH 28080, 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)-imidazo[1,2*a*]pyridine; WT, wild type.

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The studies with the Glu^{820} mutants (*18, 19, 21, 26, 27*) indicated that a single mutation gives only limited information on the significance of this residue in the mechanism of action of the enzyme. Therefore, a series of mutants targeted to Glu^{795} were prepared and characterized. This should give us the information whether the Glu^{795} residue is involved in K^+ and/or H^+ binding, and if neutralization of this residue also results in a constitutively active enzyme.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. All DNA manipulations were carried out according to standard molecular biology techniques described by Sambrook et al. (*28*). The rat gastric H^+,K^+-ATPase α-subunit cDNA (*29*) was digested with *Bgl*II and ligated into the *Bam*HI site of the pFastbacdual vector (Life Technologies, Breda, The Netherlands), which is located behind the polyhedrin promotor. The *β*-subunit cDNA of rat gastric H^+,K^+-ATPase (*29*) was digested with *Bam*HI and ligated into the *Bbs*I site behind the p10 promotor of the same pFastbacdual vector already containing the α-subunit. Site-directed mutagenesis was performed using the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI). The target mutagenic primer introduced the desired mutation in the α-subunit together with the introduction of a silent mutation, hereby creating or deleting a specific restriction site useful for the detection of the mutants. After selection and subcloning of the mutated α-subunit in the pFastbacdual vector, all mutants were checked by sequence analysis.

Generation of Recombinant Viruses. Competent DH10bac *Escherichia coli* cells (Life Technologies, Breda, The Netherlands), harboring the baculovirus genome (bacmid) and a transposition helper vector, were transformed with the pFastbacdual transfer vector containing the different (mutant) cDNAs. Upon transposition between the Tn7 sites in the transfer vector and the bacmid, recombinant bacmids were selected and isolated (*30*). Subsequently, insect Sf9 cells were transfected with recombinant bacmids using Cellfectin reagent (Life Technologies, Breda, The Netherlands). After a 3 day incubation period, recombinant baculoviruses were harvested and used to infect Sf9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

Preparation of Sf9 Membranes. Sf9 cells were grown at 27 °C in 100 mL spinner flask cultures as described by Klaassen et al. (*29*). For production of H^+,K^+-ATPase, $(1.0-1.5) \times 10^6$ cells·mL^{-1} were infected at a multiplicity of infection of 1−3 in the presence of 1% v/v ethanol (*31*) and incubated for 3 days using Xpress medium (Biowittaker, Walkersville, MD) containing 0.1% w/v pluronic F-68 (Sigma Bornem, Belgium) as described by Swarts et al. (*19*). Sf9 cells were harvested by centrifugation at 2000*g* for 5 min. After resuspension at 0 °C in 0.25 M sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0), the membranes were sonicated twice for 30 s at 60 W (Branson Power Co., Denbury, CT). After centrifugation for 30 min at 10000*g*, the supernatant was recentrifuged for 60 min at 100000*g* at 4 °C. The pelleted membranes were resuspended in the above-mentioned buffer and stored at −20 °C.

Protein Determination. Protein concentrations were determined with the modified Lowry method described by Peterson (*32*) using bovine serum albumin as a standard.

Western Blotting. Protein samples from the membrane fraction were solubilized in SDS−PAGE sample buffer and separated on SDS-gels containing 10% acrylamide according to Laemmli (*33*). For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidenefluoride membranes (Millipore Corp., Bedford, MA). The α-subunit of the gastric H^+,K^+-ATPase was detected with the polyclonal antibody HKB (*34*).

ATPase Activity Assay. The SCH 28080 sensitive ATPase activity was determined using a radiochemical method (*35*). For this purpose 0.6−5 μg of Sf9 membranes was added to 100 μL of medium, which contained 10 μM [γ-^{32}P]ATP (specific activity 100−500 mCi·$mmol^{-1}$), 1.2 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM EDTA, 0.1 mM ouabain, 1 mM NaN_3, 25 mM Tris-HCl (pH 7.0), and varying concentrations of KCl. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 μL of 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid, and after 10 min at 0 °C, the mixture was centrifuged for 10 s (10000*g*). To 0.2 mL of the clear supernatant, containing the liberated inorganic phosphate ($^{32}P_i$), was added 3 mL of OptiFluor (Canberra Packard, Tilburg, The Netherlands), and the mixture was analyzed by liquid scintillation analysis. In general, blanks were prepared by incubating in the absence of membranes. The H^+,K^+-ATPase activity is presented as the difference of the activity in the absence and presence of 100 μM SCH 28080. The activity in the presence of SCH 28080 is due to the endogenous ATPase activity present in Sf9 membranes.

ATP Phosphorylation Capacity. ATP phosphorylation was determined as described before (*18, 19, 31*). Sf9 membranes were incubated at 0 °C (10−50 μg) or 21 °C (1−5 μg) in 50 mM Tris−acetic acid (pH 6.0), 1.2 mM $MgCl_2$ in a volume of 50 μL. After 30−60 min preincubation, 10 μL of 0.6 μM [γ-^{32}P]ATP was added and incubated for 10 s at 0 or 21 °C. The reaction was stopped by adding ice-cold 5% trichloroacetic acid in 0.1 M phosphoric acid, and the phosphorylated protein was collected by filtration over a 0.8 μm membrane filter (Schleicher & Schuell, Dassel, Germany). After repeated washing, the filters were analyzed by liquid scintillation analysis. From our previous work (*21*) we know that in our expression system about 1.5% of the total protein was the recombinant wild-type gastric H^+,K^+-ATPase, based on an ELISA method. If all recombinant ATPase should be active, one would theoretically reach a phosphorylation level of 100 pmol/mg of protein, assuming one phosphorylation site per $\alpha\beta$ oligomer. We find, however, 5 pmol/mg of protein (see Results); this means that a considerable part of the recombinant H^+,K^+-ATPase is inactive. However, the results we present in this paper would not change if the ratio between active and inactive H^+,K^+-ATPase changed because we only look to the characteristics of the active fraction of our protein.

Dephosphorylation Studies. After ATP phosphorylation, the reaction mixture was diluted from 60 to 500 μL with nonradioactive ATP (final concentration 10 μM; to prevent rephosphorylation with radioactive ATP), in 50 mM Tris−acetic acid (pH 6.0) and 0−100 mM K^+. The mixture was further incubated for 3 s at 21 °C. Thereafter, the reaction was stopped as described above, and the residual phosphorylation level was determined.

Chemicals. Cellfectin, competent DH10bac *Escherichia coli* cells, and all enzymes used for DNA cloning were

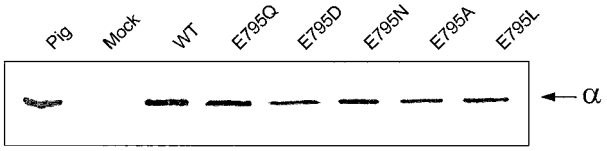

FIGURE 1: Western blot of Glu^{795} mutants of the gastric H^+,K^+-ATPase. Membranes (10−20 μg) isolated from Sf9 cells infected with a baculovirus containing either the H^+,K^+-ATPase wild type or the Glu^{795} mutants of the α-subunit were blotted. The presence of the α-subunit was detected using polyclonal antibody HKB (*45*). H^+,K^+-ATPase isolated from pig gastric mucosa and membranes from mock infected cells are shown as controls.

purchased from Life Technologies Inc. (Breda, The Netherlands). [γ-^{32}P]ATP (3000 Ci·mmol^{-1}; Amersham, Buckinghamshire, U.K.) was diluted with nonradioactive Tris−ATP (pH 6.0 or 7.0) to a specific radioactivity of 20−100 Ci·mmol^{-1}. SCH 28080, kindly provided by Dr. C. D. Strader, Schering-Plough, Kenilworth, NJ, was dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2% v/v ethanol. The antibody HKB was a gift from Dr. M. Caplan (Yale).

Analysis of Data. The $K_{0.5}$ value is defined as the concentration of effector (K^+) giving the half-maximal activation. All data are presented as mean values with standard error of the mean. Differences were tested for significance by means of the Student's *t*-test.

RESULTS

Figure 1 shows a Western blot of a series of gastric H^+,K^+-ATPase mutants, in which Glu^{795} was replaced by five other amino acids. The α-subunit of the recombinant (mutant) protein present in the Sf9 membrane fractions was detected using the HKB antibody (*34*). All (mutant) H^+,K^+-ATPase α-subunits showed the same apparent molecular weight as the α-subunit present in membrane fractions isolated from pig gastric mucosa (*36*) and absent in membrane preparations isolated from mock infected Sf9 cells. Western blotting indicated that the β-subunit was present both in a carbohydrate-free and in a core-glycosylated form (not shown), similarly as previously reported (*29*).

ATPase Activity. To measure K^+-stimulated ATPase activity, we used a suboptimal (10 μM) ATP concentration since higher ATP concentrations result in a relatively higher increase of the background ATPase activity (*21*). The SCH 28080 insensitive ATPase activity of the wild-type enzyme was in the same range as the activity in membranes isolated from mock infected cells: 120 ± 20 nmol of P_i·(mg of protein)$^{-1}$·h^{-1} (*19*). Addition of up to 30 mM K^+ had no effect on the ATPase activity in the presence of 100 μM SCH 28080.

The ATPase activity of the wild-type enzyme (Figure 2A) could be stimulated by K^+, reaching a maximal activity at 0.9 mM K^+. The $K_{0.5}$ value for K^+ stimulation of ATPase activity was 0.07 ± 0.01 mM (Table 1). At K^+ concentrations above 0.9 mM, the ATPase activity decreased due to a shift of the enzyme from the E_1 to the E_2-K^+ form. The K^+-stimulated ATPase activity could be completely inhibited by 100 μM SCH 28080.

The E795Q mutant (Figure 2B) also showed a biphasic activation curve. The rising part of this curve suggests that the apparent K^+ affinity of the E795Q mutant was higher than that of the wild-type enzyme. The K^+-induced shift to the E_2-K^+ form, represented by the decreasing part of the curve, suggests a reduced K^+ sensitivity. These opposite effects of K^+ in different parts of the reaction cycle explain the broader activation curve of the E795Q mutant as compared to that of the wild type. At higher K^+ concentrations (0.9−90 mM), the inhibition with 100 μM SCH 28080 was not complete.

Table 1: Properties of the SCH 28080 Sensitive H^+,K^+-ATPase Activity Measured in the Membrane Preparations Isolated from Infected Sf9 Cells[a]

mutant	*n*	basal ATPase act.	max ATPase act.	$K_{0.5}$
wild type	4	33 ± 8	211 ± 14**	0.07 ± 0.01
E795Q	4	82 ± 24[a]	225 ± 33**	0.03 ± 0.01[b]
E795D	4	10 ± 2[a]	24 ± 5[b,**]	1.6 ± 0.2[b]
E795N	4	4 ± 3[a]	26 ± 8[b,**]	5.1 ± 0.3[b]
E795L	4	84 ± 15[a]	86 ± 18[a]	−
E795A	4	52 ± 6	110 ± 29[a,*]	2.3 ± 0.4[b]

[a] Basal activity represents the SCH 28080 sensitive (0.1 mM) specific H^+,K^+-ATPase activity in the absence of K^+ and is expressed as nmol of P_i·(mg of protein)$^{-1}$·h^{-1}. The maximal ATPase activity represents the specific H^+,K^+-ATPase activity at optimal K^+ concentrations (see Figure 2) and has the same units as the basal ATPase activity. $K_{0.5}$ indicates the K^+ concentration (in mM KCl) needed for a half-maximal K^+ stimulation. The data are given as the mean ± SE for 4 independent experiments. [a] and [b] represent comparison of the mutants with the wild-type enzyme, whereas * and ** represent comparison between the basal and maximal, SCH 28080 sensitive, H^+,K^+-ATPase activity. [a] and *: $P < 0.05$; [b] and **: $P < 0.01$.

The E795L (Figure 2E) and E795A (Figure 2F) mutants showed an ATPase activity in the absence of added K^+. For the E795L mutant, addition of K^+ had no stimulatory effect on the ATPase activity, whereas the ATPase activity of the E795A mutant could be further stimulated at rather high K^+ concentrations. Increasing K^+ concentrations resulted in a decrease of the ATPase activity of both mutants. The mutants E795D (Figure 2C) and E795N (Figure 2D) could only be stimulated at high K^+ concentrations. The maximal ATPase activity of both mutants was very low as compared to that of the wild-type enzyme.

Table 1 summarizes the SCH 28080 sensitive ATPase activities measured in the membranes isolated from infected Sf9 cells. The basal activity is described as the SCH 28080 sensitive ATPase activity present in the absence of added K^+ ions. The E795Q and E795L mutants showed a significantly increased SCH 28080 sensitive basal ATPase activity, while that of the E795A mutant was slightly increased compared to that of the wild-type enzyme. Two other mutants, E795D and E795N, however, showed a significantly decreased SCH 28080 sensitive ATPase activity compared to the wild-type enzyme. The E795Q mutant revealed an increased K^+ sensitivity, the E795L mutant was totally insensitive for K^+ ions, and the other mutants showed a decreased K^+ sensitivity as compared to the wild-type enzyme.

ATP Phosphorylation. Phosphorylation of the gastric H^+,K^+-ATPase occurs after addition of Mg^{2+}-ATP (*35*), resulting in the formation of an acid-stable phosphorylated intermediate. All mutants showed a phosphorylated α-subunit similarly to that of the wild-type enzyme, which was revealed as a 100 kDa band upon autoradiography after SDS−PAGE of H^+,K^+-ATPase containing Sf9 membranes (data not shown).

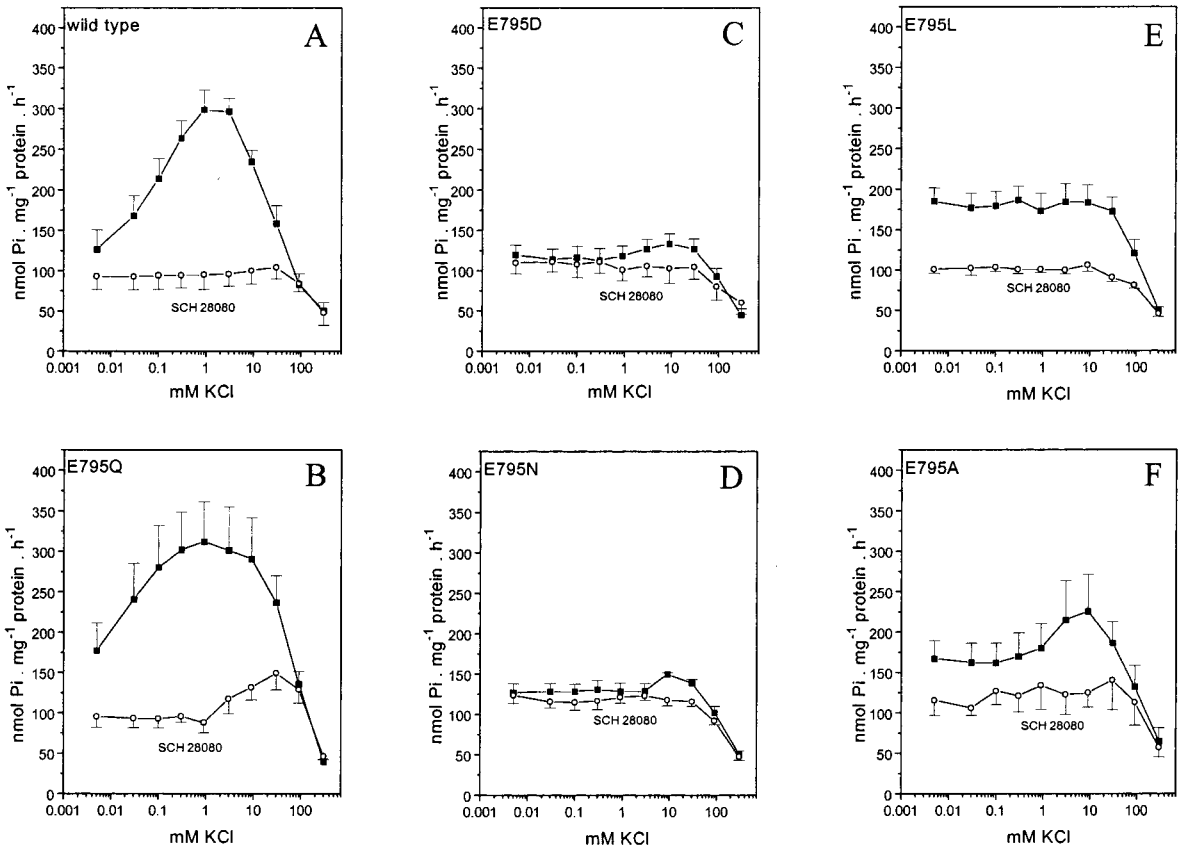

FIGURE 2: Effects of K^+ and SCH 28080 on the ATPase activity. (A) Wild type, (B) E795Q, (C) E795D, (D) E795N, (E) E795L, and (F) E795A. Membranes were prepared, and the ATPase activity in the presence of the indicated K^+ concentration was determined as described under Experimental Procedures. ATPase activity is given both in the absence (■) and in the presence (○) of 100 μM SCH 28080. Mean values are given for four independent membrane preparations; bars represent SE.

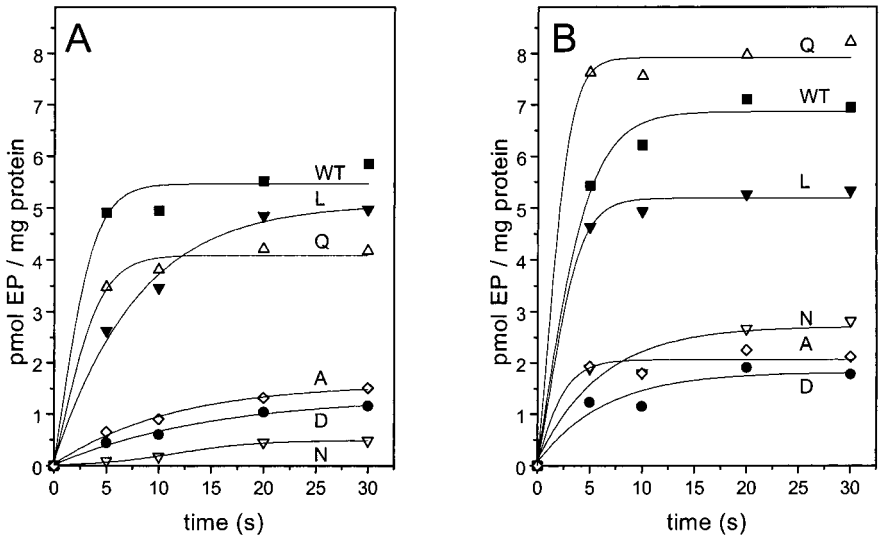

FIGURE 3: Time dependence of ATP phosphorylation of (mutant) gastric H^+,K^+-ATPase. Sf9 membranes (1 μg) were preincubated at 0 °C (panel A) or 21 °C (panel B) with 0.2 mM EDTA, 1.2 mM $MgCl_2$, and 50 mM Tris−acetic acid (pH 6.0). After phosphorylation with 0.1 μM [γ-^{32}P]ATP at the indicated temperature and time, the phosphorylation level was determined and corrected for that of the mock membranes. The following symbols are used: wild type (WT, ■); E795Q (Q, △); E795D (D, ●); E795N (N, ▽); E795L (L, ▼); and E795A (A, ◇). Representative for three experiments.

Figure 3 shows the results of ATP phosphorylation experiments performed at 0 and 21 °C at pH 6.0 with the membranes isolated from infected Sf9 cells of the wild-type enzyme and H^+,K^+-ATPase mutants for 0−30 s. Table 2 summarizes the 10 s phosphorylation levels of the recombinant (mutant) enzymes measured at 0 and 21 °C. At 0 °C, only the wild-type enzyme and the E795Q mutant reached a steady-state phosphorylation level in 10 s (Figure 3A); at 21 °C, this was reached for all mutants within 10−20 s (Figure 3B). At 21 °C, all mutants (except E795Q) showed a significantly increased 10 s steady-state phosphorylation level compared to that determined at 0 °C. The steady-state phosphorylation levels of the wild-type enzyme and the E795Q and E795L mutants were at both temperatures

Table 2: Phosphorylation Levels of Recombinant Gastric H^+,K^+-ATPase and Its Mutants at 0 and 21 °C[a]

		phosphorylation level		
mutant	*n*	0 °C	21 °C	apparent ATP affinity
wild type	3/5/4	3.86 ± 0.36	4.88 ± 0.35[a]	10.2 ± 0.2
E795Q	3/3/2	3.37 ± 0.69	4.45 ± 0.34	24.5 ± 1.4*
E795D	3/5/3	0.61 ± 0.25	1.31 ± 0.16[b]	31.2 ± 6.2*
E795N	3/5/2	0.45 ± 0.21	1.14 ± 0.14[b]	79.7 ± 2.1**
E795L	3/3/3	3.48 ± 0.28	4.99 ± 0.24[b]	28.6 ± 0.8**
E795A	3/5/2	1.10 ± 0.30	2.29 ± 0.37[a]	48.8 ± 1.9**

[a] Phosphorylation (10 s) was carried out as described under Experimental Procedures. Phosphorylation levels are expressed as pmol of EP/mg of protein and are given as the mean ± SE of 3−5 experiments. These values were corrected for the phosphorylation level measured on the membranes isolated from mock infected cells [0.64 ± 0.15 (n = 4) pmol of EP/mg of protein at 0 °C and 1.29 ± 0.07 (n = 3) pmol of EP/mg of protein at 21 °C]. The apparent ATP affinities (in nM ATP) determined in a 10 s phosphorylation experiment at 21 °C using different ATP concentrations are shown in column 5 as the mean of 2−4 experiments ± SE. [a] and [b] represent comparison of 21 °C with 0 °C, whereas * and ** represent comparison of the mutants with the wild type. [a] and *: $P < 0.05$; [b] and **: $P < 0.01$. *n*: 0 °C/21 °C/ATP affinity.

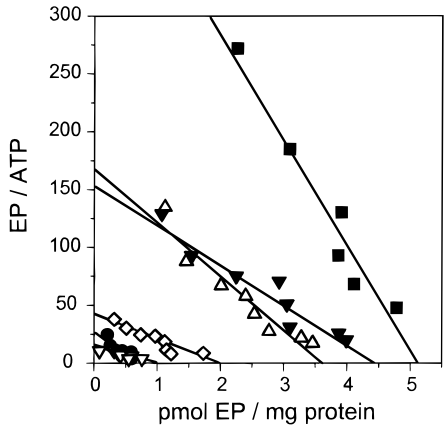

FIGURE 4: Scatchard plot of the ATP dependence of the phosphorylation level of the wild-type enzyme and the E795 mutants. A 10 s phosphorylation was performed, with 1 μg of protein and with 8−200 nM [γ-^{32}P]ATP at 21 °C as described under Experimental Procedures. The phosphorylation level (EP) was determined and expressed as picomoles per milligram of protein, plotted on the *x*-axis, whereas on the *y*-axis the ratio of EP over free ATP is plotted. The following symbols are used: wild type (■); E795Q (△); E795D (●); E795N (▽); E795L (▼); and E795A (◇). Representative for two to four experiments.

significantly higher than those of the other mutants.

ATP Affinity. The ATP dependence of the 10 s phosphorylation level (at 21 °C and pH 6.0) of the gastric H^+,K^+-ATPase preparations is shown in Figure 4. Table 2 (last column) shows the calculated apparent ATP affinities for 2−4 independent measurements. For the wild-type enzyme, an apparent ATP affinity of 10.2 ± 0.2 nM was determined. The apparent ATP affinities of the E795Q and E795L mutants were significantly reduced to 24.5 ± 1.4 and 28.6 ± 0.8 nM, respectively. For the E795D, E795N, and E795A mutants, the apparent ATP affinities decreased by a factor of 3−8 compared to the wild-type enzyme (31.2 ± 6.2, 79.7 ± 2.1, and 48.8 ± 1.9 nM, respectively).

Dephosphorylation Studies. For the wild-type enzyme, the stimulatory effect of K^+ on the ATPase activity is due to stimulation of the hydrolysis rate of the phosphorylated

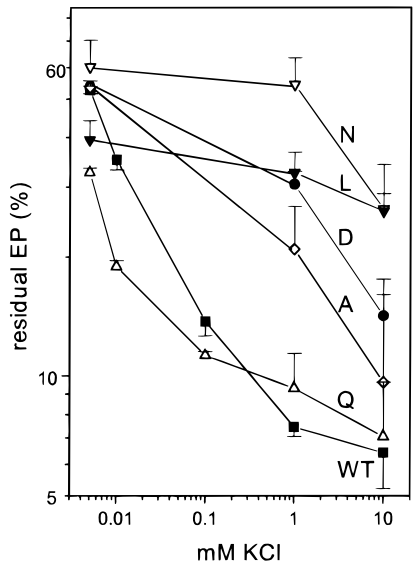

FIGURE 5: Dephosphorylation of the phosphorylated intermediate of the wild-type enzyme and the E795 mutants. Membrane preparations were phosphorylated for 10 s with 0.1 μM [γ-^{32}P]-ATP at 21 °C as described under Experimental Procedures. Dephosphorylation was started by addition of excess nonradioactive ATP in the presence of increasing K^+ concentrations. Three seconds after addition of the dephosphorylation mix, the reaction was stopped by addition of ice-cold TCA, and phosphorylation levels were determined. The results obtained with the (mutant) H^+,K^+-ATPase were corrected for those of mock infected cells. The residual phosphorylation level was expressed as a percentage of the phosphorylation level before starting the dephosphorylation step. The following symbols are used: wild type (WT, ■); E795Q (Q, △); E795D (D, ●); E795N (N, ▽); E795L (L, ▼); and E795A (A, ◇). The results presented are the mean values ± SE for two to three independent preparations.

intermediate. To check whether this is also the case for the mutants, we performed dephosphorylation experiments after 10 s phosphorylation at 21 °C. Dephosphorylation rates of these preparations were determined as the reduction of the phosphorylated intermediate levels in 3 s upon addition of increasing K^+ concentrations (Figure 5).

For the wild-type enzyme and the mutants E795D, E795N, and E795A, the residual phosphorylation levels in the absence of added K^+ [2] were similar and varied between 53 and 60%. The E795Q and E795L mutants showed under these conditions a significantly increased dephosphorylation, resulting in residual phosphorylation levels of 33 ± 1% (n = 3; $P < 0.01$) and 39 ± 5% (n = 2; $P < 0.01$), respectively. The high basal dephosphorylation of the E795L mutant has to be an inherent property of this mutant, since addition of 10 mM K^+ has only minor effects on the residual phosphorylation level. Addition of up to 100 μM K^+ present in the dephosphorylation medium of both the wild-type and the E795Q mutant resulted in a rapidly decreased residual phosphorylation level. In the presence of 10 μM K^+, the wild-type enzyme showed a residual phosphorylation level of 35 ± 2% (n = 2), whereas the E795Q mutant showed a significantly lower residual phosphorylation level of 19 ± 1% (n = 2; $P < 0.01$). It is likely that the relatively high dephosphorylation rate of the E795Q mutant in the absence of added K^+ is due to the increased apparent K^+ affinity of

[2] Flame photometry measurements indicated that the dephosphorylation medium contained about 5 μM K^+.

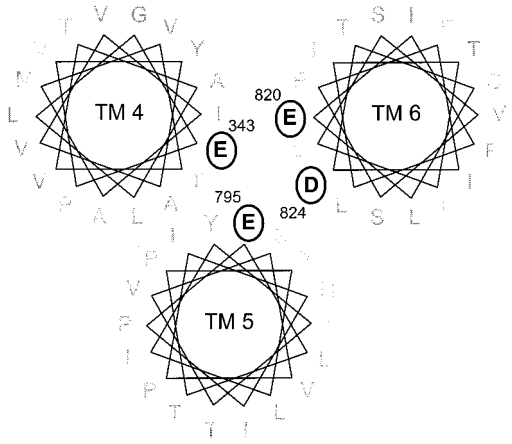

FIGURE 6: Hypothetical model of the cation binding pocket of gastric H^+,K^+-ATPase. The experimental data are consistent with a model where Glu^{345} (TM 4), Glu^{795} (TM 5), and Glu^{820} and Asp^{824} (TM 6) are involved in cation binding. These three transmembrane domains are projected as helical wheels in which the hypothetical liganding amino acid residues are plotted toward the center of the cation binding pocket.

this mutant. For the E795A, E795D, and E795N mutants, 1 mM K^+ was insufficient for a complete dephosphorylation of the phosphorylated intermediate. For these mutants, only a higher K^+ concentration (10 mM) enhanced the dephosphorylation process. This indicates that these mutants have a decreased K^+ sensitivity compared to that of the wild-type enzyme.

DISCUSSION

The role of negatively charged residues present in the fourth, fifth, and sixth transmembrane domains of gastric H^+,K^+-ATPase has been described recently (*18*−*22*, *26*, *27*). We previously observed only some minor activity changes upon replacement of Glu^{795}, present in the fifth transmembrane domain, by Gln (*21*). However, the use of different substitutions of Glu^{795}, presented in this study, revealed an essential role of this residue in gastric H^+,K^+-ATPase function. The described results can be divided into three parts. First, mutagenesis on Glu^{795} revealed that this residue plays a role in K^+ activation; however, not the negative charge but the carbonyl group of Glu^{795} is essential. Second, mutagenesis of this residue resulted for some mutants in a completely or partly constitutive H^+,K^+-ATPase activity. Finally, mutagenesis decreased the apparent ATP affinities, which might indicate a possible role of Glu^{795} in H^+-stimulated phosphorylation.

Effects on K^+ Sensitivity. Figure 6 shows a hypothetical model of the cation binding pocket of wild-type gastric H^+,K^+-ATPase, in which at least four negatively charged residues play an essential role: Glu^{345} (*20*), Asp^{824} (*21*), Glu^{820} (*18*, *19*, *21*, *22*, *26*), and Glu^{795} (this paper). The (mutant) H^+,K^+-ATPase preparations will be discussed on the basis of the measured K^+ sensitivities.

We first discuss the wild-type enzyme and the E795Q mutant, although the latter showed even an increased K^+ sensitivity, both for the ATPase reaction as well as for the dephosphorylation process. Furthermore, this E795Q mutant needed more K^+ for inhibition of the ATPase activity than the wild-type enzyme. This can be explained by assuming that for the E795Q mutant the $E_1 \leftrightarrow E_2$ equilibrium is shifted more to the left, so that more K^+ is needed for a conversion into the E_2 form.

The E795D, E795N, and E795A mutants showed in the ATPase reaction as well as in the dephosphorylation reaction a strongly decreased K^+ sensitivity. The E795L mutant did not show a K^+-stimulated ATPase activity nor a K^+-stimulated dephosphorylation. In conclusion, it seems that on position 795 a carbonyl group located on a specific position plays a pivotal role in K^+ activation, and is sufficient for full enzyme activity. This carbonyl group can originate either from a glutamate or from a glutamine residue.

Mutagenesis of the corresponding residue in Na^+,K^+-ATPase to a Gln showed similar K^+ sensitivities as compared to the wild-type enzyme (*23*). However, Nielsen et al. showed that the E779Q mutant occluded only one Tl^+ per mole of enzyme, whereas the wild-type enzyme could occlude two Tl^+ ions (*37*). Upon replacement of the corresponding residue by an Ala, the apparent K^+ affinity decreased similarly as with the E795A mutant described in this paper (*23*−*25*). Others suggested that this corresponding E779A mutant in Na^+,K^+-ATPase is not directly involved in ion binding and dephosphorylation (*9*). They proposed that Glu^{779} may be a part of the access channel determining the voltage dependence of ion transport by Na^+,K^+-ATPase (*9*). When in the Na^+,K^+-ATPase an Asp was present on this position, the ATPase activity was still 50% (*37*), which seems contradictory to an abolished high-affinity occlusion of Rb^+ and Tl^+.

Constitutive Activation. Constitutive activation is defined as a SCH 28080 sensitive ATPase activity in the absence of K^+. This constitutive activation has been described previously for some mutants of Glu^{820} (*19*). This was explained by a high basal and K^+-independent dephosphorylation rate of the phosphorylated intermediate. It is assumed that the negative charge present in the wild-type enzyme (Figure 6) inhibits the dephosphorylation process. This inhibition can be overcome by addition of K^+, which neutralizes the negatively charged pocket and hereby stimulates the dephosphorylation of the phosphorylated intermediate.

The E795L mutant showed both in the ATPase as well as in the dephosphorylation reaction a constitutive activity that could not be enhanced by addition of K^+ ions. The constitutive activation of the E795L mutant is similar to that observed with the E820Q and E820N mutants, which also showed a K^+ independent activity (*19*). From these experiments, the hypothesis has been put forward that the empty K^+ binding pocket inhibits the dephosphorylation process. K^+ ions can stimulate the dephosphorylation process by a charge neutralizing effect. Some mutants, E820Q, E820N (*19*), and E795L (this report), simulate the K^+-bound state which results in an activation of the dephosphorylation process in the absence of K^+ ions.

The E795Q mutant showed in the absence of added K^+ both a significantly increased ATPase activity as well as a higher dephosphorylation rate. This is apparently due to a higher K^+ sensitivity, as discussed above. The E795A mutant showed a small increase in the ATPase activity in the absence of K^+ compared to the wild-type enzyme. Previously, similar results have been published by our group on the E820A mutant (*19*). We concluded from these results that due to the small size of the Ala side chain the other liganding groups in the cation binding pocket can still play a role in K^+ binding. In this case, however, a much higher K^+ concentra-

tion was needed. On the other hand, we were not able to detect a higher spontaneous dephosphorylation rate for this mutant. The absence of a spontaneous dephosphorylation of the E795A mutant, whereas there is a constitutive ATPase activity, can be explained by assuming that the conformational energy needed for a spontaneous activity of this mutant is sufficient at 37 °C but not at 21 °C. A similar difference was observed with some Glu^{820} mutants (*19*), which showed a constitutive ATPase activity, but only an increased dephosphorylation rate at 21 °C and not at 0 °C.

The wild-type enzyme as well as the mutants E795D and E795N showed no constitutive activity. The wild-type enzyme and the E795D mutant both contain a negatively charged residue, and thus no constitutive activation is expected. In the E795N mutant, the negative charge has been neutralized, but due to the enlarged distance of this residue to the center of the cation binding pocket, this neutralization is probably insufficient to stimulate the dephosphorylation reaction constitutively.

Some mutants of the corresponding residue of Na^+,K^+-ATPase also showed an increased ATP hydrolysis in the absence of K^+. This phenomenon has been described for the corresponding Ala mutant (*9, 24, 25*), although these findings were interpreted by supposing that Na^+ stimulates the dephosphorylation process (*9*). For the SR Ca^{2+}-ATPase, it has been demonstrated that replacement of the corresponding residue Glu^{771} by a Lys, hereby converting the negative into a positive charge, results in a significantly increased dephosphorylation rate (*17*). This favors our hypothesis that negative charges in an empty cation binding pocket inhibit the dephosphorylation process, which can be overcome by the binding of K^+.

Effects on Phosphorylation and Apparent ATP Affinity. The wild-type enzyme and the mutants E795Q and E795L showed high steady-state phosphorylation levels and phosphorylation rates and rather high apparent ATP affinities. The E795A, E795D, and E795N mutants showed a 3−8 times lower apparent ATP affinity as compared to that of the wild-type enzyme.

The ATP binding site for P_2-type ATPases is located in the large intracellular loop between transmembrane domains 4 and 5 (*38*−*41*). The H4−H5 loop of Na^+,K^+-ATPase expressed in *E. coli* still shows ATP binding similar to that of the fully expressed wild-type enzyme (*42, 43*). These observations indicate that it is unlikely that mutagenesis of Glu^{795} in M5 of the gastric H^+,K^+-ATPase affects ATP binding directly. The Post−Albers scheme (*44*) indicates that the ratio between H^+ and K^+ concentrations determines the equilibrium between the E_1 and E_2 conformations of the enzyme. A decreased H^+ affinity might result in a shift of the equilibrium to the E_2 form, which could explain the lower apparent ATP affinity. Similar findings were reported recently for the E820D mutant (*26*). These findings suggest that the same cation binding pocket, although in different conformations, might be involved in both inward and outward cation transport.

Comparable observations were made with Na^+,K^+-ATPase. Mutagenesis of the corresponding Glu^{779} residue to an Asp, Ala, or Gln revealed that this residue is involved not only in K^+ binding but also in Na^+ binding (*23*−*25*). Also for SR Ca^{2+}-ATPase it is thought that the corresponding residue is involved in Ca^{2+}-dependent phosphorylation (*14, 17*).

In conclusion, the results presented in this report indicate that the Glu^{795} residue, present in the fifth transmembrane domain of the catalytic α-subunit of gastric H^+,K^+-ATPase, is indeed involved in K^+-stimulated dephosphorylation and K^+-stimulated ATPase activity. In contrast to Glu^{820}, it is not primarily the negative charge but the carbonyl group of glutamic acid on position 795 which is essential for full enzymatic activity. Furthermore, this carbonyl group has to be in a particular position, since both the ATPase activities and the apparent K^+ affinities of the E795D and E795N mutants were considerably lower than those of the wild-type enzyme and the E795Q mutant. Mutants neutralizing the cation binding pocket can transfer conformational energy to the phosphorylation domain and thereby stimulate the dephosphorylation process in the absence of K^+. In addition, mutagenesis of the Glu^{795} residue leads to decreased apparent ATP affinities. These changes mainly affect phosphorylation kinetics, which indicate that Glu^{795} not only is involved in K^+ binding but also might play a role in H^+ binding.

REFERENCES

1. Lutsenko, S., and Kaplan, J. H. (1995) *Biochemistry 34*, 15607−15613.
2. Pedersen, P. L., and Carafoli, E. (1987) *Trends Biochem. Sci. 12*, 146−150.
3. Capasso, J. M., Hoving, S., Tal, D. M., Goldshleger, R., and Karlish, S. J. D. (1992) *J. Biol. Chem. 267*, 1150−1158.
4. Arguello, J. M., and Kaplan, J. H. (1991) *J. Biol. Chem. 266*, 14627−14635.
5. Arguello, J. M., and Kaplan, J. H. (1994) *J. Biol. Chem. 269*, 6892−6899.
6. Goldshleger, R., Tal, D. M., Moorman, J., Stein, W. D., and Karlish, S. J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6911−6915.
7. Van Huysse, J. W., Jewell-Motz, E. A., and Lingrel, J. B. (1993) *Biochemistry 32*, 819−826.
8. Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L., and Lingrel, J. B. (1995) *J. Biol. Chem. 270*, 2993−3000.
9. Arguello, J. M., Peluffo, R. D., Feng, J. N., Lingrel, J. B., and Berlin, J. R. (1996) *J. Biol. Chem. 271*, 24610−24616.
10. Kuntzweiler, T. A., Arguello, J. M., and Lingrel, J. B. (1996) *J. Biol. Chem. 271*, 29682−29687.
11. Pedersen, P. A., Rasmussen, J. H., Nielsen, J. M., and Jørgensen, P. L. (1997) *FEBS Lett. 400*, 206−210.
12. Vilsen, B., Ramlov, D., and Andersen, J. P. (1997) *Ann. N.Y. Acad. Sci. 834*, 297−309.
13. Andersen, J. P., and Vilsen, B. (1995) *FEBS Lett. 359*, 101−106.
14. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1989) *Nature 339*, 476−478.
15. Andersen, J. P., and Vilsen, B. (1992) *J. Biol. Chem. 267*, 19383−19387.
16. Vilsen, B. (1993) *FEBS Lett. 333*, 44−50.
17. Andersen, J. P. (1994) *FEBS Lett. 354*, 93−96.
18. Hermsen, H. P. H., Swarts, H. G. P., Koenderink, J. B., and De Pont, J. J. H. H. M. (1998) *Biochem. J. 331*, 465−472.
19. Swarts, H. G. P., Hermsen, H. P. H., Koenderink, J. B., Schuurmans Stekhoven, F. M. A. H., and De Pont, J. J. H. H. M. (1998) *EMBO J. 17*, 3029−3035.
20. Asano, S., Tega, Y., Konishi, K., Fujioka, M., and Takeguchi, N. (1996) *J. Biol. Chem. 271*, 2740−2745.
21. Swarts, H. G. P., Klaassen, C. H. W., De Boer, M., Fransen, J. A. M., and De Pont, J. J. H. H. M. (1996) *J. Biol. Chem. 271*, 29764−29772.
22. Asano, S., Matsuda, S., Tega, Y., Shimizu, K., Sakamoto, S., and Takeguchi, N. (1997) *J. Biol. Chem. 272*, 17668−17674.

23. Feng, J. N., and Lingrel, J. B. (1995) *Cell. Mol. Biol. Res. 41*, 29−37.
24. Vilsen, B. (1995) *Biochemistry 34*, 1455−1463.
25. Koster, J. C., Blanco, G., Mills, P. B., and Mercer, R. W. (1996) *J. Biol. Chem. 271*, 2413−2421.
26. Hermsen, H. P. H., Swarts, H. G. P., Koenderink, J. B., and De Pont, J. J. H. H. M. (1999) *Biochim. Biophys. Acta 1416*, 251−257.
27. Swarts, H. G. P., Hermsen, H. P. H., Koenderink, J. B., Willems, P. H. G. M., and De Pont, J. J. H. H. M. (1999) *Mol. Pharmacol. 55*, 541−547.
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Klaassen, C. H. W., Van Uem, T. J. F., De Moel, M. P., De Caluwé, G. L. J., Swarts, H. G. P., and De Pont, J. J. H. H. M. (1993) *FEBS Lett. 329*, 277−282.
30. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) *J. Virol. 67*, 4566−4579.
31. Klaassen, C. H. W., Swarts, H. G. P., and De Pont, J. J. H. H. M. (1995) *Biochem. Biophys. Res. Commun. 210*, 907−913.
32. Peterson, G. L. (1983) *Methods Enzymol. 91*, 95−106.
33. Laemmli, U. K. (1970) *Nature 227*, 680−685.
34. Gottardi, C. J., and Caplan, M. J. (1993) *J. Biol. Chem. 268*, 14342−14347.
35. Swarts, H. G. P., Klaassen, C. H. W., Schuurmans Stekhoven, F. M. A. H., and De Pont, J. J. H. H. M. (1995) *J. Biol. Chem. 270*, 7890−7895.
36. Swarts, H. G. P., Van Uem, T. J. F., Hoving, S., Fransen, J. A. M., and De Pont, J. J. H. H. M. (1991) *Biochim. Biophys. Acta 1070*, 283−292.
37. Nielsen, J. M., Pedersen, P. A., Karlish, S. J. D., and Jørgensen, P. J. (1998) *Biochemistry 37*, 1961−1968.
38. Pedersen, P. A., Rasmussen, J. H., and Jørgensen, P. L. (1996) *Biochemistry 35*, 16085−16093.
39. Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L., and Lingrel, J. B. (1995) *J. Biol. Chem. 270*, 16206−16212.
40. Andersen, J. P. (1995) *Biosci. Rep. 15*, 243−261.
41. Møller, J. V., Juul, B., and Le Maire, M. (1996) *Biochim. Biophys. Acta 1286*, 1−51.
42. Gatto, C., Wang, A. X., and Kaplan, J. H. (1998) *J. Biol. Chem. 273*, 10578−10585.
43. Obsil, T., Merola, F., Lewit-Bentley, A., and Amler, E. (1998) *FEBS Lett. 426*, 297−300.
44. Rabon, E. C., and Reuben, M. A. (1990) *Annu. Rev. Physiol. 52*, 321−344.
45. Blostein, R., Zhang, R. P., Gottardi, C. J., and Caplan, M. J. (1993) *J. Biol. Chem. 268*, 10654−10658.

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