Phosphorylation of the epithelial Na\textsuperscript{+} channel (ENaC) has been suggested to play a role in its regulation. Here we demonstrate that phosphorylating the carboxyl termini of the \(\beta\) and \(\gamma\) subunits facilitates their interactions with the ubiquitin ligase Nedd4 and inhibits channel activity. Three protein kinases, which phosphorylate the carboxyl termini of \(\beta\) and \(\gamma\)ENaC, have been identified by an in vitro assay. One of these phosphorylates \(\beta\)Thr-613 and \(\gamma\)Thr-623, well-conserved C-tail threonines in the immediate vicinity of the PY motifs. Phosphorylation of \(\gamma\)Thr-623 has also been demonstrated in vivo in channels expressed in Xenopus oocytes, and mutating \(\beta\)Thr-613 and \(\gamma\)Thr-623 into alanine increased the channel activity by 3.5-fold. Effects of the above phosphorylations on interactions between ENaC and Nedd4 have been studied using surface plasmon resonance. Peptides having phospho-threonine at positions \(\beta\)613 or \(\gamma\)623 bind the WW domains of Nedd4 two to three times better than the non-phosphorylated analogues, due to higher association rate constants. Using a number of different approaches it was demonstrated that the protein kinase acting on \(\beta\)Thr-613 and \(\gamma\)Thr-623 is the extracellular regulated kinase (ERK). It is suggested that an ERK-mediated phosphorylation of \(\beta\)Thr-613 and \(\gamma\)Thr-623 down-regulates the channel by facilitating its interaction with Nedd4.

Active Na\textsuperscript{+} reabsorption in kidney collecting duct, distal colon, lung, and exocrine glands is mediated by an apical amiloride-blockable Na\textsuperscript{+} channel (1–3). The channel is composed of three homologous subunits, denoted \(\alpha\), \(\beta\), and \(\gamma\)ENaC (Epithelial Na\textsuperscript{+} Channel).\textsuperscript{1} Its cell surface expression is determined by interactions of the C-tails of \(\beta\) and \(\gamma\) with the ubiquitin ligase Nedd4. The WW domains of Nedd4 bind to the proline-rich PY motifs on \(\beta\) and \(\gamma\)ENaC, leading to channel ubiquitination, internalization, and degradation (4, 5). A central role of ENaC in maintaining salt and water balance has been conclusively demonstrated by identifying genetic diseases associated with mutations in channel subunits, as well as by the phenotypic analysis of ENaC knockout mice (for review see Refs. 1–3).

ENaC’s activity is also controlled by a number of hormones such as the mineralocorticoid aldosterone, the anti-diuretic peptide vasopressin, and insulin (1, 3). Previous studies have suggested the involvement of protein phosphorylation in these mechanisms. The serine/threonine kinase \(sgh\) (serum and glucocorticoid-dependent kinase) is induced by aldosterone and can activate the channel upon co-expression in Xenopus oocytes (6–8). This response was recently found to involve phosphorylation of Nedd4-2 by \(sgh\) (9, 10). The response of A6 cells to both aldosterone and insulin requires activation of phosphoinositide 3-kinase (11, 12). In addition, aldosterone and insulin, as well as intracellular signaling components such as protein kinases C and A, increase the in vivo phosphorylation of the carboxyl termini of both \(\beta\) and \(\gamma\)ENaC (13).

We have recently demonstrated phosphorylation of the carboxyl termini of ENaC subunits, expressed as glutathione S-transferase (GST) fusion proteins by crude cytosolic fractions (14). The current study characterizes conserved residues phosphorylated in the carboxyl termini of ENaC subunits, explores their physiological role, and identifies the kinase involved. The data indicate that an extracellular regulated kinase (ERK)-dependent phosphorylation of \(\beta\)Thr-613 and \(\gamma\)Thr-623 may be important in controlling interactions between the channel and Nedd4.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA and Proteins**—The carboxyl termini of the rat \(\beta\) and \(\gamma\)ENaC (\(\beta\)557–638 and \(\gamma\)564–650) were subcloned downstream GST in the bacterial expression vector pGEKX3 as described in a previous study (14). cDNA constructs expressing fusion proteins between GST and the three WW domains of rat Nedd4 were kindly provided by D. Rotin (Hospital for Sick Children, Toronto, Canada) and are described in a previous study (5). GST fusion proteins were expressed in a protease-deficient Escherichia coli strain and purified on glutathione beads as detailed previously (14). Functional expression in Xenopus oocytes was done using ENaC clones in pSPORT-1, obtained from B. C. Rossier (Institute of Pharmacology, University of Lausanne).

Immunoprecipitation of phosphorylated ENaC was performed using a hemagglutinin A (HA)-tagged \(\beta\) and \(\gamma\) construct kindly provided by B. Schwappach (Zentrum für Molekulare Biologie, Universität Heidelberg). The HA epitope was introduced in the ecto domains of these subunits at a position shown before not to affect channel activity (15). Point mutations in the various constructs were introduced using a QuikChange site-directed mutagenesis kit and verified by sequencing.

**Extraction, Fractionation, and Assay of Cytosolic Protein Kinases**—Cytosol was extracted from rat distal colon and fractionated by ion exchange chromatography using the following protocol. Rats (Wistar, 9–11 weeks old) were sacrificed by cervical dislocation. The distal colon was excised, cut open, and rinsed first in phosphate-buffered saline and then in buffer A composed of: 50 mM \(\beta\)-glycerophosphate, pH 7.3, 1.5 mM...
EGTA, 1.0 mM EDTA, 1.0 mM dithioreitol, and 0.1 mM de-activated sodium orthovanadate (16). The epithelial cells were scraped off the connective tissue using a glass slide and suspended in buffer A + a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1.0 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2.0 μg/ml pepstatin A). Cells were washed by two centrifugation and then resuspended on ice by sonication (3 × 5 s). Cell homogenates were centrifuged for 30 min at 30,000 × g, and the supernatants were collected and further fractionated by ion exchange chromatography. 10 μg of protein in 50 μl of buffer A was loaded onto a MonoQ column (Amersham Biosciences, Inc.) at a rate of 1 ml/min. Bound proteins were eluted at the same rate by a linear NaCl gradient (0–100 mM NaCl) at 4 °C. Ninety aliquots were collected, stored at 4 °C, and assayed for kinase activity within 24 h.

Chinese hamster ovary (CHO) cells stably transfected with the human insulin receptor were kindly provided by Dr. Yehiel Zick (Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel). Cells were cultivated in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F12 supplemented with 10% fetal calf serum and 1% glutamine. After achieving 80% confluency, the medium was replaced by serum-free Dulbecco’s modified Eagle’s medium for a period of 24–36 h. Serum-depleted cells were incubated with 100 nM insulin for 5–120 min in the presence and absence of various inhibitors. They were washed in ice-cold phosphate-buffered saline, harvested, and suspended in buffer A + protease inhibitors. Cells were disrupted by 7-s sonication on ice. The homogenates were centrifuged for 60 min at 15,000 × g, and the supernatants were collected.

Rat colon cytosol MonoQ fractions, whole CHO cytosol, and purified activated ERK2 were used in an in vitro phosphorylation assay of GST-ENaC fusion proteins. The fusion proteins were immobilized on glutathione-agarose beads and suspended in buffer A. Aliquots of 47 μl were mixed with 50-μl volumes of cytosolic fractions, whole cytosol (~1 mg/ml protein), or purified ERK2 (1.5 ng/ml, specific activity of 0.45 mmol/min/mg), all in buffer A. Phosphorylation was initiated by the addition of 3 μl of ATP mixture composed of: 1.8 μl of 1 mM MgCl2, 0.9 μl of 0.2 mM ATP, and 0.3 μl of [γ-32P]ATP (10 μCi/ml, 3000 Ci/mmol). Suspensions were shaken for 30 min at 30 °C, pelleted, and washed several times in HB1B buffer (20 μM HEPES, pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 25 mM MgCl2, and 0.05% Triton X-100). Pellets were suspended in Laemmli sample buffer, boiled for 5 min, resolved on 12% SDS-PAGE, and exposed to a PhosphorImager plate and/or x-ray film.

Specific binding of cytosolic kinases to ENaC C-tail fusion proteins was determined by co-precipitation, as described previously (17). In brief, fusion proteins immobilized on glutathione beads were incubated with CHO C-terminal cytosolic fractions or purified kinases for 1 h, at 4 °C. Incubation was done in a binding buffer composed of 150 mM NaCl, 22 mM HEPES, pH 7.7, 2 mM MgCl2, 0.075% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM EDTA, 0.1 mM sodium orthovanadate, and protease inhibitors. The beads were sedimented and washed twice in HB1B buffer, a third wash in buffer A, and a final wash in a kinase assay buffer (20 mM HEPES, pH 7.7, 20 mM MgCl2, 20 mM β-glycerophosphate, 1 mM dithioreitol, and 0.1 mM sodium orthovanadate). Beads were suspended in 30-μl volumes of the above kinase assay buffer, and phosphorylation was initiated by the addition of 2 μM ATP plus 2 μCi of [γ-32P]ATP.

Western Blotting—30-μl aliquots of rat colon MonoQ fractions or whole CHO-T cytosol were resolved on 10% SDS-PAGE, blotted onto nitrocellulose, and blocked with 5% low fat milk. Samples were probed with either anti-ERK (1:20,000) or anti-phospho ERK (1:5000). Both anti-ERK antibodies were obtained from Sigma-Aldrich Fine Chemicals. Blots were overlaid with horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, 1:10,000), and binding was detected by enhanced chemiluminescence.

BIAcore Experiments—Binding of the WW domains of Nedd4 to β and γ PV peptides was monitored by surface plasmon resonance in a BIAcore 2000 sensor, as described previously (18). In brief, peptides serving as substrates were immobilized on the sensor chip (SA, BIAcore, Uppsala Sweden) through amino termini biotin residues. In each chip, the peptides to be compared were immobilized on two of the channels. Free biotin and γ peptide carrying the mutation Y628A were immobilized on the other two channels and served as negative controls. The three WW domains of rat Nedd4 were expressed as GST fusion proteins and used as analytes. They were suspended in HBS buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 3.4 mM EDTA, and 0.005% P20) and injected at a flow rate of 20 μl/min. Binding was monitored simultaneously in all four channels for at least 4 min and terminated by the application of HBS buffer without analyte (disassociation phase). The chip was regenerated by a subsequent injection of 10 μl of HBS + 0.05% SDS and extensively washed in HBS, and then the next analyte was applied. Each observation was confirmed by at least three measurements in two different chips.

Functional Expression and Phosphorylation in Xenopus Oocytes—The ENaC-mediated Na+ current was determined in the oocyte expression system as described before (8, 14). In brief, stage V–VI oocytes were incubated with cRNA mixtures containing 2.5 ng of each of the ENaC subunits. Oocytes were incubated at 17 °C in a medium that contained 96 mM NaCl and 10 μM amiloride. Electrophysiological measurements were performed 48–72 h later by means of a two-electrode voltage clamp technique. Channel activity was determined as the amiloride-sensitive current amplitudes monitored at −100 mV. Phosphorylation was examined in oocytes injected with cRNA mixtures in which either β or γ were HA-tagged. Groups of ~40 ENaC-expressing oocytes were incubated for 4 h with 3.3 μCi of orthophosphate (32P). A mixture of orthovanadate (0.5 mM) and H2O2 (1 mM) was added in the last 60 min of the incubation to stimulate endogenous kinases. Another aliquot of ~40 injected oocytes was metabolically labeled by a 2-day incubation with 100 μCi of [35S]methionine. Oocytes labeled with 32P or 35S were washed and homogenized in buffer A + protease inhibitors, and membranes were isolated by centrifugation through a sucrose cushion. Membranes were solubilized in 1% Triton X-100 in buffer A and centrifuged for 5 min at 11,000 × g to remove insoluble material. Aliquots of ~200 μl of detergent-soluble membrane protein extracts were incubated for 12–16 h at 4 °C with 2 μg of a mouse monoclonal anti-HA antibody (clone 12CA5, Roche Molecular Biochemicals) and then for another 2 h with protein A-Sepharose beads. The beads were sedimented, washed twice in buffer A + 0.1% Triton X-100, and a third time in buffer A + 0.5 mM LiCl. Immunopellets were suspended in SDS sample buffer, resolved on 8% SDS-PAGE, and assayed for radioactivity.

RESULTS

In Vitro and in Vivo Phosphorylation of ENaC—Cytosol extracted from rat distal colon has been used to identify protein kinases capable of phosphorlyating ENaC subunits. The colonic tissue was selected, because it is relatively homogenous and has high Na+ channel abundance. Proteins were fractionated on a MonoQ column, and fractions were tested for their ability to phosphorylate fusion proteins having the carboxyl termini of γ ENaC. Fig. 1 depicts a typical assay demonstrating the existence of at least three protein kinase-enriched fractions phos- phorylating GST-γ. The first peak of protein kinase activity was eluted around fractions 38–41 (~0.07–0.09 mM NaCl) and is characterized in this study. A second peak, seen around fraction 59 (~0.19 mM NaCl), was found to phosphorylate residue yThr-630 (data not shown). Hence, it is likely to be the same residue detected in crude DE-52 fractions reported before (14). A third, major peak was seen around fractions 74–77 (~0.25–0.27 mM NaCl) and will be described elsewhere.

Initial experiments have used mutagenesis to identify ENaC residues phosphorylated by the kinase eluted around fraction 40. Phosphorylation of GST-γ appeared to be on Thr-623, and mutating this site to Ala residue completely inhibited incorpo-
Phosphorylation of β and γENaC Facilitates Nedd4 Binding

Fig. 2. In vitro phosphorylation of GST-γ by fractionated cytosol. A, GST fusion proteins containing wild type and mutated γ C-tail were phosphorylated by fraction 40. Autoradiogram and Coomassie Blue staining are depicted. B, phosphorylation of GST-γ and γT623A was done following co-precipitation of the kinase and the fusion protein as described under “Experimental Procedures.” C, sequence alignment of the PY motifs in β and γENaC. Usually the fusion protein was partly degraded resulting in two or more bands in Coomassie Blue staining. Only the upper (full-length) band had the phosphorylation site.

Fig. 3. Functional expression and phosphorylation of ENaC in Xenopus oocytes. A, oocytes were injected with cRNA mixtures in which one of the subunits was HA tagged. They were labeled with either [35S]methionine or 32Pi as described under “Experimental Procedures.” HA-tagged proteins were immunoprecipitated, resolved electrophoretically, and assayed for 35S and 32P radioactivity. B, oocytes were injected with wild type and mutated subunits. Channel activity was determined as the amiloride-blockable current at −100 mV and expressed as a fraction of the mean current in oocytes injected with wild type ENaC. Data were accumulated from four different frogs (means ± S.E., n = 26–38 oocytes).

It was further demonstrated that the protein kinase eluted in fraction 40 tightly binds to the γ C-tail and can be co-precipitated with it. In this assay, GST-γ immobilized on glutathione beads was incubated with the above cytosolic fraction in the absence of ATP. The beads were washed several times and then incubated with [γ-32P]ATP with no added protein kinases. Substantial phosphorylation of the fusion protein can take place under these conditions only if some of the kinase is precipitated with its substrate. As shown in Fig. 2B, GST-γ could be effectively phosphorylated following such co-precipitation, and the phosphorylation occurred on Thr-623.

Next, it was determined that the above phosphorylation takes place also in Xenopus oocytes expressing the three ENaC subunits. Accordingly, HA-tagged wild type and mutated subunits were translated in the oocyte system and metabolically labeled with either [35S]methionine or [32P]. Orthovanadate and H2O2 were added during the incubation with [32P] to achieve activation of endogenous protein kinases. Immunoprecipitation of HA-tagged β and γ demonstrated phosphorylation of both subunits (Fig. 3A). Mutating γThr-623 into an Ala residue significantly decreased incorporation of [32P] into this subunit without affecting labeling by [35S]methionine. In three different experiments using 40 oocytes each, the mutation of Thr-623 lowered the [32P]/[35S] ratio of the immunoprecipitated subunit by 47 ± 15%. Thus, Thr-623 is one of the γ residues phosphorylated in the oocyte system. Phosphorylation of the β subunit on the other hand, was not significantly affected by mutating Thr-613 into Ala. Thus, this residue is either not phosphorylated in oocytes or its phosphorylation is masked by γ incorporation into other residues. This may be similar to the phenomenon described above where mutating βThr-613 into Ala had a minor effect on the overall β phosphorylation by fractionated cytosol, even though it is clearly one of the phosphorylated residues (see subsequent experiments in CHO-T cells). Expressing the double mutant βT613A/γT623A in oocytes resulted in macroscopic Na+ currents that were 3.5-fold higher than that evoked by the wild type channel (Fig. 3B). Mutating the same residues into glutamic acid had a smaller but nevertheless significant stimulatory effect. Thus, γThr-623 and/or βThr-613 have a functional role and their mutation largely activates the channel. However, because mutation to a neutral or negatively charged amino acid evoked the same response, it is not certain that this activation is due to the inability to phosphorylate threonine 613/623. 

Effects of Phosphorylation on the Channel/Nedd4 Interactions—γThr-623 and βThr-613 are well-conserved residues located immediately before the PY motifs (Fig. 2C). Their phosphorylation could affect the channel by altering its interaction with Nedd4. Assessment of such a mechanism has been done by determining association of the WW regions of Nedd4 with phosphorylated and non-phosphorylated PY peptides using BIAcore (18). Accordingly, various β and γ peptides, listed in Table I,
were synthesized and attached to streptavidin-coated sensor chips via an amino-terminal biotin moiety. The three WW domains of rat Nedd4, expressed as GST fusion proteins, were passed over the chip and binding was recorded as a change in refractive index. A typical sensogram-monitoring interactions of WW2 to γPY peptides is illustrated in Fig. 4A. Binding of the recombinant protein to the phosphorylated peptide (γ pThr-623) was 2- to 3-fold higher than that recorded for the non-phosphorylated analogue (γ). No significant binding was observed with a peptide carrying a point mutation that impairs Nedd4-ENaC interactions (γ Y628A), or to free biotin. The small rapid signal seen in these channels is probably due to bulk or nonspecific effects. Therefore, in all subsequent experiments the signal obtained in the biotin channel was subtracted from readings in other channels to obtain ENaC-specific signals. We have also tested a γ peptide with phospho-threonine at position 630, shown to be phosphorylated by another cytosolic fraction (14). This peptide (γ pThr-630) appeared to bind WW2 at a rate that was close to that of the wild type peptide (Fig. 4B).

The above protocol was repeated for different concentrations of all three WW domains using both β and γ peptides (Fig. 5). All three WW domains were found to bind better to PY peptides that have phospho-threonine at position βThr13 or γThr-23. The experiment of Fig. 5 also confirms a previous observation that WW2 and WW3 bind PY sequences much better than WW1 (19, 20). Additional competition experiments summarized in Fig. 6, further establishes the above findings. In this experiment the WW3 fusion protein was preincubated in solution with different non-biotinylated peptides, and then applied to the sensor chip. The competing soluble peptide largely inhibited association of WW3 with the immobilized peptide, and phospho-peptides were more effective competitors than the non-phosphorylated analogues. This result was observed irrespective of the immobilized substrate (β or γ) or analyte (WW2 or WW3, data not shown). It demonstrates that the phosphorylated peptides better associate with the WW fusion proteins also in solution; i.e. it rules out the possibility that their larger binding reflects differences in peptide immobilization or packing on the chip.

Although the above data clearly show effects of phosphorylation on the interactions between WW proteins and PY peptides, kinetic analysis of these effects was not trivial. As reported previously, the association could not be well fitted to simple 1:1 Langmuir binding and a considerable fraction of the response was attributed to a very slow and non-saturable component (18). This phase was preceded by a faster saturable component that was particularly noticeable at high concentrations of the analytes. This phenomenon was independent of the flow rate (20–75 μl/min) and hence not likely to reflect mass transfer effects. Data could in principle be fitted to a sum of two exponentials, but the fit was satisfactory only for the interaction of β and β pThr-613 with WW3. In this case, the faster event had the characteristics of a simple 1:1 binding and its apparent association rate constants (kₐ) showed the expected linear dependence on the analyte concentration (Fig. 7). The dissociation rate constants (kₖ) were concentration independent. The kinetics parameters extracted from this analysis, and the equilibrium constants calculated as ratios of the association and dissociation rate constants, are summarized in Table II. The phospho-β peptide was found to have ~4-fold higher affinity toward WW3, due to an increased association rate constant. The second, slower phase could not be analyzed kinetically, but this component too was markedly affected by phosphorylation.

**Identifying the Protein Kinase Phosphorylating βThr-613 and γThr-623**—Next, we studied the identity of the cytosolic kinase eluted in fraction 40, which phosphorylates γThr-623. The presence of multiple proline residues near the phosphorylation site suggested involvement of a proline-directed kinase, e.g. a member of the MAPK family such as ERK, p38, or c-Jun amino-terminal kinase (JNK). In particular, involvement of ERK seemed likely, because the sequence PXTP is a typical ERK phosphorylation motif (21). This possibility was assessed in the experiments depicted in Figs. 8 and 9. First, the active rat colon fractions were tested for the presence of this kinase using antibodies against total and active (phosphorylated) ERK. Indeed, both ERK1 and -2 were present in the same MonoQ fractions that incorporate ³²P into γThr-623, and the abundance of active ERK in the various fractions correlated with their phosphorylation activity (Fig. 8, top and middle panels). Other experiments tested phosphorylation of the β and γ fusion proteins by purified activated ERK2. This protein kinase effectively incorporated ³²P from ATP into both GST-β

---

**TABLE I**

| Peptide sequence | Specifications |
|------------------|---------------|
| I P G T P P P N Y D S L R L Q | βPY peptide |
| I P G pT P P P N Y D S L R L Q | βPY peptide with pThr-613 |
| V P G T P P P R Y N T L R L D | γPY peptide |
| V P G pT P P P R Y N T L R L D | γPY peptide with pThr-623 |
| V P G T P P P R Y N pT L R L D | γPY peptide with pThr-630 |
| V P G T P P P R A N T L R L D | γPY peptide with Y628A |

---

**FIG. 4.** BIAcore detection of the binding of WW2 to γ PY peptides. A, equal amounts of γ, γ pThr-623, γ Y628A, and biotin were immobilized on four channels of the same chip. WW2 (final concentrations of 4.8 μM) was injected at time zero (first arrow), and its association was monitored for ~4 min. The analytes were then replaced by BBS buffer (second arrow), and dissociation was recorded for a similar period of time. B, same as in A except that the phospho-peptide tested was γ pThr-630.
FIG. 5. Effect of phosphorylation on the binding of WW domains to PY peptides. Increasing concentrations of the three WW fusion proteins were applied to sensor chips having phosphorylated and non-phosphorylated β and γ peptides. After each application, the chip was perfused with 10 μl of HBS + 0.05% SDS and extensively washed with HBS. The analyte concentrations used for β were 0.3, 0.6, 1.2, 2.4, and 4.8 μM. For γ, concentrations of 0.075, 0.15, 0.3, 0.6, and 1.2 μM were applied.
absence of competing peptide.

Data are presented as fraction of WW3 binding in the presence of phosphorylated peptide.

Experiments. Data are presented as fraction of WW3 binding in the presence of phosphorylated peptide.

FIG. 6. Competition of phosphorylated and non-phosphorylated peptides. Aliquots of WW3 (0.8 μM) were preincubated with either dithionite or different peptides (250 μM). The mixtures were applied onto the sensor chip, and binding to phosphorylated β peptide was monitored. A, sequential recordings following the injections of: WW3, WW3 + β, WW3 + βp, WW3 + γ, WW3 + γp, and WW3. Injection of the analyte (first arrow) was followed by injection of HBS (second arrow). The chip was then regenerated with 10 μl of HSB + 0.05% SDS (third arrow) and finally equilibrated with HBS (fourth arrow).

Subsequent experiments have used CHO cells overexpressing the insulin receptor (CHO-T) as a means to achieve regulated activation of MAPK and phosphatases. Hence, they provide a convenient tool for examining the role of these kinases in the phosphorylation of GST-γ. We found that the C-tail fusion proteins of ENaC were phosphorylated mainly by two protein kinases present in CHO-T; ERK and casein kinase 2 (CK2). Therefore, it was possible to study phosphorylation of βγ613 and γ623 using whole, non-fractionated, cytosol providing that CK2 was completely inhibited by heparin or that its major phosphorylation sites (γThr-599, βSer-631) were mutated. Under these conditions phosphorylation of GST-β and γ by whole CHO-T cytosol took place only on residues βThr-613 and γThr-623, respectively (Fig. 10A).

FIG. 7. Kinetic analysis of the association of WW3 to phosphorylated and non-phosphorylated β peptides. Association curves were fitted to the sum of two exponentials. The association rate constants (k1) were calculated from slopes of the best-fitted lines from plots of k1 versus the analyte concentration (Fig. 7, r = 0.99). The dissociation rate constants (k2) are means ± S.D. of values obtained for different concentrations of the analyte. The equilibrium constants (K1) are k1/k2.

$k_a = 3.0 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$

$k_d = 0.029 \pm 0.003 \mu\text{M}$

$k_a = 5.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$

$k_d = 0.021 \pm 0.003 \mu\text{M}$

TABLE II

| Analyte | Substrate | k_a (M\(^{-1}\)s\(^{-1}\)) | k_d (s\(^{-1}\)) | K_d (μM) |
|---------|-----------|-----------------------------|-----------------|----------|
| WW3     | β pT613   | 2.3 \times 10^4             | 0.026 ± 0.010   | 1.1      |
| WW3     | β         | 5.0 \times 10^4             | 0.021 ± 0.003   | 4.2      |

FIG. 8. Western blotting with anti-ERK antibody. Fractions 34–50 eluted from the MonoQ column were used for the following three assays: a, phosphorylation of GST-γ (top panel); b, blotting with an anti-phospho ERK antibody (middle panel); c, blotting with an antibody that interacts with both phosphorylated and non-phosphorylated ERK (lower panel).

Hence, they provide a convenient tool for examining the role of these kinases in the phosphorylation of GST-γ. We found that the C-tail fusion proteins of ENaC were phosphorylated mainly by two protein kinases present in CHO-T; ERK and casein kinase 2 (CK2). Therefore, it was possible to study phosphorylation of βγ613 and γ623 using whole, non-fractionated, cytosol providing that CK2 was completely inhibited by heparin or that its major phosphorylation sites (γThr-599, βSer-631) were mutated. Under these conditions phosphorylation of GST-β and γ by whole CHO-T cytosol took place only on residues βThr-613 and γThr-623, respectively (Fig. 10A).

FIG. 9. Phosphorylation of GST-β and γ by ERK2. A, wild type and mutated GST-β and γ were phosphorylated by purified activated ERK2 (final concentration, 0.7 ng/ml; specific activity, 0.45 mmol of phosphate/min/mg of protein). B, phosphorylation was done following co-precipitation of the fusion proteins and ERK2 as described under “Experimental Procedures.”

Hence, they provide a convenient tool for examining the role of these kinases in the phosphorylation of GST-γ. We found that the C-tail fusion proteins of ENaC were phosphorylated mainly by two protein kinases present in CHO-T; ERK and casein kinase 2 (CK2). Therefore, it was possible to study phosphorylation of βγ613 and γ623 using whole, non-fractionated, cytosol providing that CK2 was completely inhibited by heparin or that its major phosphorylation sites (γThr-599, βSer-631) were mutated. Under these conditions phosphorylation of GST-β and γ by whole CHO-T cytosol took place only on residues βThr-613 and γThr-623, respectively (Fig. 10A).
Phosphorylation of both subunits was strongly stimulated by insulin (Fig. 10B). This response was blocked by PD98059 but slightly activated by SB203580 (Fig. 10, B and C). PD98059 is a specific inhibitor of ERK activation, interacting with the upstream kinase (MAPK kinase) (23). SB203580, on the other hand, specifically inhibits p38 MAPK. The slight increase in phosphorylation evoked by this compound probably reflects the activation of Raf-1 and a subsequent stimulation of ERK (24). The above interpretation was further confirmed by immunoblots of CHO-T cytosol with antibodies against phosphorylated and total ERK (Fig. 10D). Insulin, PD98059, and SB203580 produced the expected changes in the abundance of phosphorylated ERK without affecting the total abundance of this protein kinase.

It was further shown that the capacity of CHO-T cytosol to phosphorylate GST-γ correlates in time with the activation of ERK (Fig. 11, A and B). In this experiment, cytosol was extracted from CHO-T cells that were incubated for different periods of time with insulin. It was then assayed for the activation of ERK (using the anti-phospho-ERK antibody) and the phosphorylation of GST-γ (Fig. 11D). Phosphorylation of both subunits was strongly stimulated by insulin induced phosphorylation of β. Bar graph: means ± S.E. of three experiments using different cytosolic preparations. B, Western blotting of the cytosol from A with antibodies against phosphorylated (upper) and total (lower) ERK. C, cytosols, extracted from CHO-T cells incubated with insulin for the indicated periods of time, were utilized for a co-precipitation experiment using either GST or GST-γ. The precipitated fusion proteins were resolved by PAGE and blotted with the anti-phospho ERK antibody.

incorporation of 32P into βThr-613 and γThr-623. These residues and the prolines around them are well conserved through evolution. In fact the position βThr-613/γThr-623 is one of only three cytoplasmic serine/threonines fully conserved in all β and γENaC subunits cloned so far (the other two are βSer620/γThr-630 and βSer631/γThr-644). The following evidence suggests that the protein kinase phosphorylating βThr-613 and γThr-623 is ERK. (i) ERK is co-precipitated from whole cytosol by GST-γ and -β and phosphorylates them at γThr-623 and βThr-613. (ii) This protein kinase is eluted from the MonoQ column in the same fractions found to incorporate 32P into γThr-623 and βThr-613. (iii) Purified activated ERK2 can bind the carboxyl termini of β and γ and phosphorylate βThr-613 and γThr-623. (iv) The insulin-induced phosphorylation of GST-γ by CHO-T cytosol has a kinetic and pharmacological profile that fits well activation of ERK1/2. Because identification of ERK as the kinase phosphorylating βThr-613 and γThr-623 is based on in vitro measurements only, it is in principle possible that other kinases too may phosphorylate these residues in vivo.

It was also demonstrated that at least γThr-623 is phosphorylated in Xenopus oocytes. In addition, injecting oocytes with doubly mutated channel T613AT623A elevated the amiloride-
blockable Na\(^+\) current by 3.4 ± 0.8-fold. This is consistent with previous data showing a ~2-fold increase of Na\(^+\) current in oocytes expressing the singly mutated channel βT613A (25). Mutating these residues into glutamic acid (which may mimic phospho-threonine) failed to produce the opposite effect (Fig. 3). These data may indicate that 1) glutamic acid behaves differently than phospho-threonine in this particular case and 2) the activation observed upon mutating βThr-613 and γThr-623 is independent of their phosphorylation.

Because the phosphorylated threonine flanks the PY motifs of β and γ, we have tested the possibility that it influences interaction with Nedd4. Binding was assessed by surface plasmon resonance using phosphorylated and non-phosphorylated synthetic peptides corresponding to the PY domains. The use of synthetic peptides was required, because the GST fusion proteins were only ~10% phosphorylated. The following three criteria have established that the observed associations of GST-PP1 with the immobilized PY peptides are specific and resemble the physiological interaction between ENaC and Nedd4. (i) Binding was largely inhibited by mutating the essential tyrosine γY628 into alanine. (ii) Preincubating WW2 and 3 with β or γ peptides blocked their association to the immobilized substrate. (iii) In agreement with previous studies using other methods (19, 20), the PY peptides could bind WW2 and 3 much better than WW1.

Whereas a specific interaction between PY peptides and WW fusion proteins were readily demonstrated, their kinetic analysis turned out to be complicated. Both associations and dissociations were biphasic, and the slow phase did not saturate at the times and concentrations used. The nature of this slow phase is not clear. It may represent a slow aggregation of the fusion protein on the chip, following its initial binding to the peptide or an induced-fit phenomenon. Nevertheless, because the slow phase is also abolished by preincubation with peptides, blocked by the mutagenY628A, and not seen for WW1, it is probably physiologically relevant.

A major finding was that the phosphorylated peptides bind the WW fusion proteins more than three times more than do their non-phosphorylated analogues. This result was obtained for both β and γ and was independent of the WW domain used. Kinetic analysis of the early phase demonstrated that this elevation reflects an increase in the association rate constant with no significant change in the dissociation rate constant. Thus, the above data are consistent with the model that phosphorylation of βThr-613 and γThr-623 by ERK and maybe other kinases facilitates the ENaC-Nedd4 interaction and down-regulates the channel. Mutating these residues inhibits this interaction and increases channel activity in X. laevis oocytes. It should, however, be mentioned that recent studies revealed functional differences between Nedd4-1 used in this study and Nedd4-2, which is phosphorylated by sgk (9, 10). Thus, the situation may be a more complex one.

The above mechanism is also in agreement with the suggestion that WW domains act as binding modules for phosphoserines and threonines to help determine specificity in signaling cascades (26). The structure of a complex between WW3 and a β PY peptide in solution has been recently solved by NMR (27). According to this structure, βThr-613 is not directly involved in WW binding and is part of a region that is disordered in solution. The difference between the two sets of data may stem from the different peptide structures used in both cases.

Yet unknown is the upstream signaling pathway that mediates its effect on ENaC through the phosphorylation of β613/γ623. At least three extra- or intracellular signals known to regulate ENaC may in principle activate ERK. A first possibility is that the mechanism described in this study plays a role in a stress response and in particular in the hypotonic-induced inhibition of channels. Hypotonic shock is a well-established activator of ERK, which is of particular importance in epithelial cells that normally experience extreme osmolarities (28). A hypotonic-induced inhibition of ENaC has been demonstrated (29), and it may be mediated by phosphorylating βThr-613 and/or γThr-623. The second involves transforming growth factor β, which was shown to inhibit the aldosterone-induced Na\(^+\) conductance at a stage distal to channel transcription (30). Because transforming growth factors β1 and 2 are well-established activators of ERK (31), desensitization of the response to aldosterone may be mediated by channel phosphorylation. A third option is participation of ERK in the Ca\(^{2+}\)-induced inhibition of Na\(^+\) transport. It is well-established that epithelial cells down-regulate their apical Na\(^+\) permeability in response to increased cell Na\(^+\) and that this “feedback” inhibition is mediated by a rise in cell Ca\(^{2+}\) (1). An increase in cell Ca\(^{2+}\) translocates Nedd4 to the apical membrane and decreases cell surface expression of ENaC (32, 33). Ca\(^{2+}\) can activate ERK through either Rap or Ras (34) and, hence, mediate its inhibitory action also by a channel phosphorylation.

Other studies have related components of the Ras pathway to the regulation of ENaC by adrenal corticosteroids (35–37). These findings, however, predict effects that are opposite to those reported in the current study; i.e. an increase in Na\(^+\) conductance as a result of ERK activation. It was also reported that ERK antagonizes induction of ENaC by glucocorticoids (38, 39). In this case, regulation involves channel transcriptional and not its post-translational modification. Finally, it is possible that enhanced phosphorylation of ENaC is triggered by the inhibition of a specific phosphatase rather than the activation of a kinase. This was found to be the case in the phosphorylation-dependent ubiquitination of the cAMP response element in hypoxia (40).

In conclusion, the current study identifies a mechanism by which ERK down-regulates ENaC by enhancing channel association with Nedd4. The exact role of this process in the physiological regulation of Na\(^+\) transport awaits further studies.

Acknowledgments—We thank T. Hanoch and Z. Yao for useful discussion.

REFERENCES

1. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
2. Horisberger, J. D. (1998) Curr. Opin. Cell Biol. 10, 445–449
3. Schild, L., and Garty, H. (1997) in Cytoskeleton and Pathophysiology (Seldin, D. W., and Giebisch, G., eds) 3rd Ed., pp. 251–276, Lippincott Williams and Wilkins, Philadelphia, PA
4. Staub, O., Gauschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) EMBO J. 16, 6325–6336
5. Staub, O., Dho, S., Henry, P. C., Correa, J., Ishikawa, T., Meglade, J., and Rotin, D. (1996) EMBO J. 15, 2371–2380
6. Chen, S. Y., Bhargava, A., Mastroberardino, L., Meijer, O. C., Wang, J., Wabe, P., Firestone, G. L., Verrey, F., and Pearce, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2514–2519
7. Naray-Fejes-Toth, A., Canessa, C. M., Clevelend, E. S., Aldrich, G., and Fejes-Toth, G. (1999) J. Biol. Chem. 274, 16973–16978
8. Shigae, A., Asher, C., Latter, H., Garty, H., and Reuveny, E. (2000) Am. J. Physiol. 278, F913–F919
9. Debonneville, C., Flores, S. Y., Kamynina, E., Plant, P. J., Tauxe, C., Thomas, M. A., Munster, C., Hsu, A. P., Pratt, J. H., Horisberger, J. D., Pearce, D., Loffing, J., and Staub, O. (2001) EMBO J. 20, 7032–7039
10. Snider, P. M., Onon, D. R., and Thomas, E. C. (2002) J. Biol. Chem. 277, 5–8
11. Blazer-Yost, B. L., Punescu, T. G., Helman, S. I., Lee, K. D., and Vlahos, C. J. (1999) Am. J. Physiol. 277, C531–C536
12. Record, R. D., Froelich, L. W., Vlahos, C. J., and Blazer-Yost, B. L. (1998) Am. J. Physiol. 274, E611–E617
13. Shimkets, R. A., Lifton, R., and Canessa, C. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3301–3305
14. Chigaev, A., Lu, G., Shi, H.-K., Asher, C., Xu, R., Hutter, H., Seger, R., Garty, H., and Reuveny, E. (2001) Am. J. Physiol. 280, F1030–F1036
15. Firsov, D., Schild, L., Gauschi, I., Merillat, A. M., Schneeberger, E., and Schild, L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 15370–15375
16. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. (1990) J. Biol. Chem. 265, 11487–11494
17. Rubinfeld, H., and Seger, R. (1998) in Current Protocols in Cell Biology, John

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
Phosphorylation of \(\beta\) and \(\gamma\)ENaC Facilitates Nedd4 Binding

Wiley & Sons, Inc, New York

18. Asher, C., Chigaev, A., and Garty, H. (2001) *Biochem. Biophys. Res. Commun.* 286, 1228–1231
19. Farr, T. J., Coddington-Lawson, S. J., Snyder, P. M., and McDonald, F. J. (2000) *Biochem. J.* 345, 503–509
20. Harvey, K. F., Dinudom, A., Komwatana, P., Jolliffe, C. N., Day, M. L., Parasivam, G., Cook, D. I., and Kumar, S. (1999) *J. Biol. Chem.* 274, 12525–12530
21. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) *J. Biol. Chem.* 266, 22159–22163
22. Seger, R., Biener, Y., Feinstein, R., Hanoch, T., Gazit, A., and Zick, Y. (1995) *J. Biol. Chem.* 270, 28325–28330
23. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) *Endocrinology* 142, 153–183
24. Kalmes, A., Deou, J., Clowes, A. W., and Daum, G. (1999) *FEBS Lett.* 444, 71–74
25. Schild, L., Lu, Y., Gutschii, I., Schneeberger, E., Lifton, R. P., and Rossier, B. C. (1996) *EMBO J.* 15, 2381–2387
26. Lu, P.-J., Zhou, X. Z., Shen, M., and Lu, K. P. (1999) *Science* 283, 1325–1328
27. Kanelis, V., Rotin, D., and Forman-Kay, J. D. (2001) *Nat. Struct. Biol.* 8, 407–412
28. Tian, W., Zhang, Z., and Cohen, D. M. (2000) *Am. J. Physiol.* 279, F593–F604
29. Ji, H. L., Fuller, C. M., and Benos, D. J. (1998) *Am. J. Physiol.* 275, C1182–C1190
30. Husted, R. F., Sigmund, R. D., and Stokes, J. B. (2000) *Am. J. Physiol.* 278, F425–F433
31. Mulder, K. M. (2000) *Cytokine Growth Factor Rev.* 11, 23–35
32. Plant, P. J., Yeger, H., Staeh, O., Howard, P., and Rotin, D. (1997) *J. Biol. Chem.* 272, 32329–32336
33. Ishikawa, T., Marunaka, Y., and Rotin, D. (1998) *J. Gen. Physiol.* 111, 825–846
34. Grewal, S. S., York, R. D., and Stark, P. J. (1999) *Curr. Opin. Neurobiol.* 9, 544–553
35. Spindler, B., Mastroberardino, L., Custer, M., and Verrey, F. (1997) *Pflugers Arch.* 434, 323–331
36. Al-Baldawi, N. F., Stockand, J. D., Khalili, O. K., Yue, G., and Eaton, D. C. (2000) *Am. J. Physiol.* 279, C429–C439
37. Mastroberardino, L., Spindler, B., Forster, I., Loffing, J., Assandri, R., May, A., and Verrey, F. (1998) *Mol. Biol. Cell* 9, 3417–3427
38. Lin, H. H., Zentner, M. D., Ho, H. L., Kim, K. J., and Ann, D. K. (1999) *J. Biol. Chem.* 274, 21544–21554
39. Zentner, M. D., Lin, H. H., Wen, X., Kim, K. J., and Ann, D. K. (1998) *J. Biol. Chem.* 273, 30770–30776
40. Taylor, C. T., Furuta, G. T., Synnestvedt, K., and Colgan, S. P. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 12091–12096
Interactions of β and γENaC with Nedd4 Can Be Facilitated by an ERK-mediated Phosphorylation

Haikun Shi, Carol Asher, Alexander Chigaev, Yuval Yung, Eitan Reuveny, Rony Seger and Haim Garty

*J. Biol. Chem.* 2002, 277:13539-13547.  
doi: 10.1074/jbc.M111717200 originally published online January 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111717200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 38 references, 19 of which can be accessed free at http://www.jbc.org/content/277/16/13539.full.html#ref-list-1