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Redistribution of a 120 kDa Phosphoprotein in the Parietal Cell Associated with Stimulation

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Abstract. When rabbit isolated gastric glands were stimulated via the cyclic AMP pathway, a phosphorylated protein band of about 120 kDa (pp120) was markedly increased in the apical membrane-rich fraction, concomitant with an increase in the amount of H,K-ATPase and the phosphorylation of the cytoskeletal protein ezrin in the same fraction. The cytosolic fraction, but not other membrane fractions, also contained a protein with common features to the membrane-bound pp120, i.e., comigration in two-dimensional gels with a pI of ~4.5, anomalous mobility in SDS-PAGE, reactivity to antibodies, and phosphorylation, indicating that these two proteins were identical. The possibility that pp120 is vinculin was completely excluded. Using antibody against pp120, this protein was found to be almost exclusively in the gastric parietal cell. Biochemical and immunohistochemical analyses suggest that pp120 exists mainly in the cytosol, and that a small part of the protein binds to the apical membrane when the parietal cell is stimulated via the cyclic AMP pathway. In the presence of histone, purified pp120 produced phosphorylation on pp120 as well as histone. The inhibitor profile of this kinase activity is not consistent with any known kinase. We conclude that pp120 is closely associated with a new type of kinase, and translocates from cytosol to the apical membrane when the parietal cell is stimulated.

Key words: Phosphoprotein — Gastric acid secretion — Parietal cell — Protein kinases — Vinculin — Rabbit

Introduction

It has been established that stimulation of gastric acid secretion occurs concomitant with a translocation of H,K-ATPase from cytoplasmic tubulovesicles to the apical plasma membrane of the parietal cell, together with the activation of K^+ and Cl^- conductances (for review, Urushidani & Forte, 1997). It is currently of great interest to discover how second messengers, such as adenosine 3′,5′-cyclic monophosphate (cAMP) or Ca^{2+}, trigger the above changes. There is a consensus that cAMP activates cAMP-dependent protein kinase(s) and consequently phosphorylates certain protein(s) (Chew, 1985); thus, it is important to identify actual substrates for protein kinases that operate in parietal cell activation. Using isolated, ^{32}P-loaded rabbit gastric glands, we previously identified two specific phosphoproteins in the apical membrane-rich fraction (Urushidani, Hanzel & Forte, 1987), the same membrane locale where primary events for activating the proton pump occur (Urushidani & Forte, 1987). Phosphorylation of these proteins was correlated with both the redistribution of H,K-ATPase from microsomes (tubulovesicles) to the apical membrane-rich fraction, and the stimulation of acid secretion via the cAMP pathway (Urushidani et al., 1987, 1989). One of the proteins, an 80 kDa phosphoprotein, has been identified as ezrin and postulated to be working as a membrane-cytoskeletal linker (Hanzel et al., 1991). We now report on the other phosphoprotein which migrated with an apparent molecular weight of 120 kDa on 7.5% polyacrylamide gel electrophoresis (pp120). We previously found that pp120 was present in the apical membrane-rich fraction obtained from stimulated glands but not from resting ones (Urushidani et al., 1987); however, it was difficult to ascertain whether stimulation of the cell (*a*) triggered the phosphorylation of pp120 in the apical membranes, or (*b*) induced the association, or translocation, of pp120 to the membranes. We show here that pp120, which is now demonstrated to be a unique protein of the parietal cell, is present as a soluble component when the cell is at rest, and that some of this protein becomes associated with the apical membrane when the

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cell is stimulated. Moreover, we also provide evidence that pp120 is closely associated with a new type of protein kinase.

Materials and Methods

Isolation of Rabbit Gastric Glands, Labeling Glands with ^{32}P and Fractionation

The procedures were identical to those previously reported (Urushidani & Forte, 1987; Urushidani et al., 1987, 1989; Hanzel et al., 1989). In brief, rabbit gastric glands were isolated according to Berglindh & Obrink (1976), and the settled glands were dispersed in 4 vol of phosphate-free medium [(in mM): 130 NaCl, 5.4 KCl, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 25 HEPES-Na, pH = 7.4, 11.1 Glucose, and BSA (2 mg/ml)] containing 37 MBq ^{32}P-orthophosphate and incubated for 40 min at 37°C. After washing to remove external ^{32}P, the glands were incubated 40 min with stimulants (0.1 mM histamine plus 50 μM isobutylmethylxanthine (IBMX) for the maximal stimulation), or an H_2-receptor blocker (0.1 mM cimetidine). The glands were then homogenized and centrifuged to produce a series of pellets: P0, 40 × *g* for 5 min (cell debris); P1, 4,000 × *g* for 10 min (low speed fraction); P2, 14,500 × *g* for 10 min; P3, 48,200 × *g* for 90 min (microsomes), and S3, supernatant (cytosol). The low-speed P1 fraction was further purified with a Ficoll-discontinuous gradient (135,000 × *g* for 2 hr) and the membranes on the top of the 18% Ficoll layer were collected as the apical membrane-rich fraction.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Conventional two dimensional electrophoresis, with the first dimension as isoelectric focusing (IEF) and the second dimension as SDS-PAGE, was according to O'Farrel (1975). Proteins in the gel were stained with 0.125% Coomassie Blue in methanol:acetic acid:water, 5:1:4, or silver (Heukeshoven & Dernick, 1985). Since pp120 drastically changes its apparent molecular weight depending upon the percentage of acrylamide, another type of two dimensional electrophoresis was employed: both dimensions are SDS-PAGE, but the gel density was increased. Namely, the samples were first run on a 6% SDS-PAGE, then each lane was cut out and equilibrated with a stacking gel buffer for 10 min. The gel strips were laid horizontally onto a second 12% polyacrylamide gel slab. In this system most of the proteins align along a diagonal, but pp120, whose apparent mobility increases as the percentage of the gel rises, clearly separates from the diagonal row of protein spots.

Peptide mapping was done according to Cleveland et al. (1977). Protein bands were cut out from the gel, fragmented, and loaded on to a second gel (linear 6–12% gradient); digestion with V8 protease from Staphylococcus aureus (Sigma) was performed in the well of the gradient gel.

For immunoblotting, the proteins were electrophoretically transferred to nitrocellulose (Gibson, 1981) using a semidry apparatus (1 mA/cm^2, for 40 min). The antigen was probed by the antibodies and visualized using appropriate second antibodies linked with peroxidase.

For the in vitro phosphorylation studies, samples were brought to 50 mM PIPES, pH 6.8, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM PMSF, 10 μM pepstatin, 0.2 mM [γ-^{32}P]ATP, and mixed with test material when desired. The mixture was incubated at 25°C for 15 min and the reaction was terminated with 10 mM EDTA. The sample was then analyzed on SDS-PAGE.

For autoradiography, Kodak X-Omat AR X-ray film was placed between a dried gel and an intensifying screen (Cronex lightening plus, DuPont) and exposed at −80°C. The autoradiogram was scanned by an image scanner (EPSON GT-6000) and densitometrically analyzed for quantification.

Phosphoaminoacid analysis was performed as previously reported (Urushidani et al., 1989) employing two dimensional electrophoresis.

Purification of pp120

Purification of denatured pp120 was performed with preparative SDS-PAGE. An aliquot (1–3 mg) of cytosolic or apical membrane-rich fraction from isolated glands or gastric mucosa was run on a 7.5% gel using a wide well; pp120 was identified using β-galactosidase as a migration marker in a side well after a quick staining/destaining. The gel strip was cut out, minced and electroeluted as described (Urushidani et al., 1989).

Purification of non-denatured pp120 was performed as follows. All the steps except lyophilization and HPLC were undertaken at 4°C. The fundic portion of rabbit mucosa was homogenized in 20 vol. of medium containing, in mM: mannitol 113; sucrose 37; PIPES 5; EDTA-Tris 0.4. The cytosolic fraction (supernatant from 100,000 × *g* for 45 min) was lyophilized and pooled until further purification. The pooled powder, corresponding to three rabbit stomachs, was reconstituted with 1/2 the original volume of chilled water, and brought to 30% saturation of $(NH_4)_2SO_4$. Any precipitate was removed by centrifugation. The supernatant was brought to 50% saturation $(NH_4)_2SO_4$ and then spun down at 20,000 × *g* to harvest the pellet. The pellet was dissolved in the minimum amount (around 20 ml) of a 5 mM Tris, 0.2 mM EDTA buffer (TE). After adding 50 μM pepstatin and 0.1 mM PMSF, the sample was dialyzed overnight against 2,000 ml of TE buffer using tubing (Spectra Pore 6) with a 25 kDa cutoff. Filaments that appeared were removed by centrifugation, and the sample was applied to a Sephadex G-150 column (Pharmacia, 3 × 120 cm) equilibrated with TE. Chromatography was run by upward elution with TE; sample collection began as soon as protein appeared and fractions were pooled until pepsin activity first appeared. The pooled sample was brought to 150 mM KCl by adding solid KCl and applied to a DEAE-Sepharose column (Pharmacia, 1.5 × 25 cm) equilibrated with 150 mM KCl in TE. After washing the column, proteins were eluted with a gradient of 200–600 mM KCl in TE (100 ml total). The fractions that eluted from 14 to 17 mmho were collected and used as a partially purified pp120 preparation (DEAE-fraction). For further purification this fraction was dialyzed against 10 mM sodium phosphate, pH 7.0. After removing the precipitates by centrifugation, the sample was applied to an hydroxyapatite HPLC column (Tonen, HA100), equilibrated with 10 mM sodium phosphate, and eluted with a gradient of 10 to 300 mM sodium phosphate, pH 7.0. By monitoring OD_{280}, the second main peak, containing almost pure pp120 protein, was collected.

Purification of Vinculin

Vinculin was partially purified from rabbit gastric smooth muscle according to the method of Feramisco & Burridge (1980), up to the step before the ion exchange column. Monoclonal antibody against chicken gizzard vinculin was purchased from ICN ImmunoBiologicals, Lisle, IL.

Preparation of Antibodies

Polyclonal antibodies were raised against non-denatured pp120 purified by HPLC. Four Wistar strain rats were injected intraperitoneally twice over a two week interval with 500 μl of an emulsion of Freund's complete adjuvant containing about 80 μg of pp120. Ten days after the last injection, about 50 μg of pp120 was injected intravenously every week for 3 weeks. Two out of 4 rats raised antibody specific to pp120

and whole blood was collected from them. Immunohistochemistry of isolated glands was performed as previously reported (Hanzel et al., 1989) with a fluorescence microscope, Nikon Diaphot 200.

Monoclonal antibodies were raised against denatured pp120 purified by electroelution from preparative SDS-PAGE. Two Balb/c mice were first immunized with 100 μl of an emulsion of complete Freund's adjuvant that contained about 20 μg of pp120 as antigen. The mice were boosted by injecting 5 to 10 μg of antigen intravenously once every 10 days for two months. Three days after the last injection, spleen cells from one of the mice were fused to the myeloma cell line P3X63Ag8.653 by standard procedures (Goding, 1983). The culture supernatants from growing clones were screened with ELISA using partially purified pp120 as antigen; all of the 196 growing clones were positive. Four clones that were shown positive by western blotting, 4/1E9, 4/2D10, 4/2B4, 4/2G9, were further cloned to monoclonal by limiting dilution. All of these clones can immunoprecipitate pp120 with protein A agarose (Repligen, MA) or Pansorbin (Calbiochem, CA) as precipitant.

Studies of phosphorylation using immunoprecipitated pp120 were performed as follows. The cytosolic fraction was brought to 1% Nonidet P-40 (NP40) and incubated with anti-pp120 monoclonal antibody at 4°C, overnight. Fixed *staphylococcus aureus* (Pharmacia) was then added, and the sample was incubated at room temperature for 1 hr, and then spun down. The pellet was washed once with 20 mM Tris, pH 7.0, 150 mM NaCl, 1% NP-40, using a 1.25 M sucrose cushion. The pellet was further washed 3 times with buffer containing 0.1% NP-40, and the final pellet was brought to 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, 1 mM [γ-^{32}P]ATP, and incubated with 1 mg/ml of histone or protamine at 37°C. The reaction mixture was then analyzed by SDS-PAGE and autoradiography.

Results

Phoshorylation of 120 kDa Region of Apical Membrane Associated with Secretory Stimulation

As previously reported (Urushidani & Forte, 1987; Urushidani et al., 1987, 1989; Hanzel et al., 1991), stimulation of gastric glands with histamine plus IBMX elicited three main changes in the apical membrane-rich fraction, namely, (i) an increment of 96 kDa protein (Fig. 1*c vs.* 1*d*), corresponding to an increase in incorporation of H,K-ATPase from tubulovesicles to apical membranes, (ii) an increase in phosphorylation of an 80 kDa protein (Fig. 1*g vs.* 1*h*), which has been identified as ezrin (Hanzel et al., 1991), and (iii) an increase in phosphorylation in the 120 kDa region (Fig. 1*g vs.* 1*h*, arrowhead). On conventional two dimensional gels (IEF/SDS-PAGE), pp120 focused around pH 4.5, and the autoradiographic density of this phosphoprotein was quantified. In addition, it was also feasible to quantify autoradiographic density from one-dimensional gels, since the acidic pp120 was the only radioactive species within the 120 kDa molecular weight region on two-dimensional gels. The autoradiographic volume density of pp120 in stimulated membranes was recorded and set at unity; density of resting membranes was then recorded as a fraction of their stimulated counterpart. This particular normalization procedure was necessary because the autoradiographic density of pp120 in the resting membrane fraction was very low and often equal to zero. Therefore, a resting/stimulated ratio less than 1.0 indicates that the density of pp120 for stimulated preparations increased compared with the resting control.

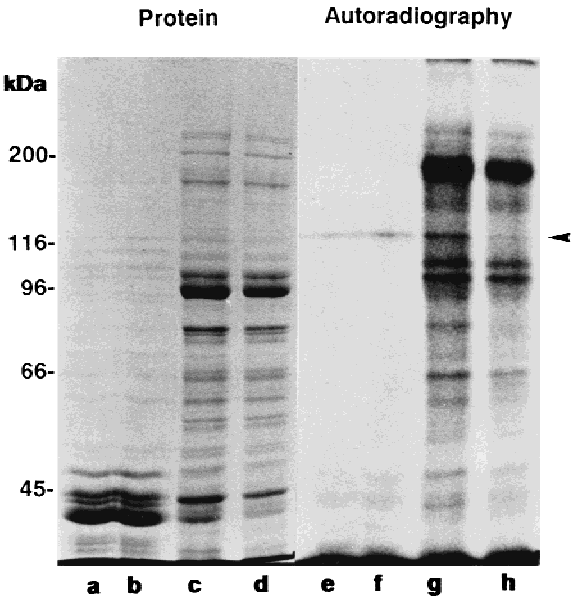

Fig. 1. SDS-PAGE of cytosol (lanes *a, b, e, f*) and apical membrane-rich fraction (lanes *c, d, g, h*) from ^{32}P-loaded, stimulated- (histamine 0.1 mM plus IBMX 50 μM; *a, c, e, g*) and resting (cimetidine 0.1 mM; *b, d, f, h*) glands. The apical membrane-rich fraction from stimulated glands is distinctive as the 96 kDa α-subunit of H,K-ATPase is enriched (lane *c* compared with *d*), and the regions of 120 kDa (pp120, arrowhead) and 80 kDa are more heavily phosphorylated than in the resting counterpart (lane *g* compared with *h*). Faint bands of protein staining, corresponding to radioactivity at pp120, can be identified in cytosol (*a, b*) from both resting and stimulated glands, as well as in the apical membrane-rich fraction from stimulated ones (*c*), but this protein band is almost invisible in the apical membrane-rich fraction from resting glands (*d*).

When histamine and IBMX were used as stimulant the resting/stimulated ratio of phosphorylation for pp120 in the apical membrane-rich fraction using the two dimensional gel system was 0.13 ± 0.07 ($N = 4$). In nine experiments using the one dimensional SDS-PAGE system the phosphorylation ratio for resting/histamine plus IBMX-stimulated glands in the 120 kDa region was calculated to be 0.23 ± 0.06 ($N = 9$). In three separate experiments, 1 mM dibutyryl cyclic AMP was used as a stimulant, and the resting/stimulated ratio obtained was 0.21 ± 0.10 ($N = 3$, from one dimensional gels). In all of the above cases, the differences between resting and stimulated were statistically significant ($P < 0.05$, paired *t*-test). However, in three experiments, when the glands were stimulated with 0.1 mM carbachol, no activity could be detected as a definite band in two cases, and very faint bands were noted in the third case for both resting and stimulated samples, giving an unreliable resting/stimu-

lated ratio of about 1.2, indicating that carbachol had no effect on the phosphorylation in the 120 kDa region.

Although the amount of protein was too low to calculate a specific activity in many of the above cases, it appeared qualitatively that radioactivity was roughly proportional to the amount of protein in the acidic pp120 spot. This suggested that the increase in phosphorylation in the 120 kDa region of stimulated apical membranes might not be due to phosphorylation of a resident membrane protein, but possibly due to the appearance, or migration, of a protein into this membrane fraction from other fraction(s). To test this hypothesis, we reviewed the autoradiography of other fractions and found a phosphoprotein with the same molecular weight in the cytosolic fraction (Fig. 1*a*,*b*,*e*,*f*). In order to confirm that the phosphoprotein in the cytosol was identical to that in the apical membrane-rich fraction, both proteins with 120 kDa molecular weight were purified from ^{32}P-labeled glands by preparative SDS-PAGE, and analyzed by two-dimensional electrophoresis (IEF/SDS-PAGE). Both preparations showed a single radioactive spot around pH = 4.5. Moreover, a mixture of the both samples gave a single spot on two-dimensional gels confirming that these two proteins, one from the apical membrane-rich fraction and the other from the cytosolic fraction, share same molecular weight and isoelectric point (*data not shown*). Finally, a comparable map of proteolytic peptide fragments was obtained for 120 kDa protein from either cytosol or the apical membrane-rich fraction (*see* Fig. 5, lanes *a* and *c*), confirming that these two proteins are identical.

Another characteristic of pp120 is that its apparent molecular weight on SDS-PAGE decreases as the concentration of acrylamide increases. Using this property, we developed a two-dimensional SDS-PAGE assay system to selectively identify pp120. The sample was first separated on a 6% acrylamide gel, and the lane was cut out and layered horizontally onto a second 12% gel. After running the second gel, most proteins aligned on the diagonal, whereas pp120, which behaved as if it were larger than expected compared to the migration of β-galactosidase (116 kDa) on 6% PAGE, but almost identical to phosphorylase b (96 kDa) on 12% PAGE, appeared as a distinct spot beneath the main protein streak. This technique enabled us to more precisely quantify pp120 in various fractions. Figure 2 shows the cytosol and apical membrane-rich fractions obtained from ^{32}P-labeled, resting and stimulated glands analyzed by this technique. It is evident that the radioactive protein, pp120 (arrowhead), is enriched in the cytosolic fraction regardless of the stimulatory state, while the protein can be found in the apical membrane-rich fraction only in the stimulated state. Densitometric analysis of these gels revealed that specific activity of pp120 was almost constant regardless of its origin and stimulatory state.

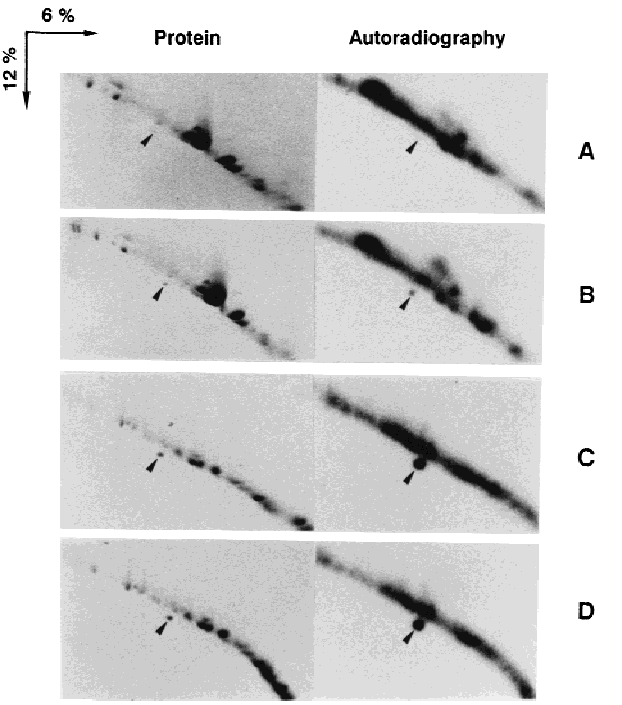

Fig. 2. Two-dimensional SDS-PAGE analysis for visualization of pp120. The apical membrane-rich fraction from resting (*A*) or stimulated (*B*) glands, or the cytosolic fraction from resting (*C*) or stimulated (*D*) glands (75 μg protein in each case) were first separated on 6% SDS-PAGE (horizontal). Each lane was then cut out and placed on top of a second 12% SDS-PAGE, and run downwards. pp120 (arrowhead) was noticeable apart from the main diagonal streak because of its anomalous mobility. Protein staining is shown on the left and the autoradiography on the right. In the cytosolic fractions, the amount of pp120, both protein and radioactivity, was the same for resting (*C*) and stimulated (*D*) glands. On the other hand, the apical membrane-rich fraction from stimulated glands (*B*) contained a considerable amount of pp120, while the fraction from resting glands (*A*) had only a trace of pp120 protein and corresponding radioactivity. Molecular weight markers are omitted; the position of the arrowhead corresponds to about 130 kDa on the horizontal scale and to about 100 kDa on the vertical scale.

From the results presented above, it could be concluded that at least a portion of pp120, which exists mainly in the cytosol as a soluble form, redistributes to the apical membrane when the cell is stimulated. To confirm this hypothesis, the cytosol and the apical membrane fraction were probed using anti-pp120 monoclonal antibody (for antibody production, *see* the later section). From the western blot in Fig. 3 (right panel) it is evident that stimulation of the glands caused the translocation of H,K-ATPase from the tubulovesicles to the apical membranes as previously reported (Urushidani & Forte, 1987). In the same preparation, the apical membrane of resting glands contained only a trace amount of pp120, whereas that of stimulated glands contained a markedly increased amount of the protein as evident from the left

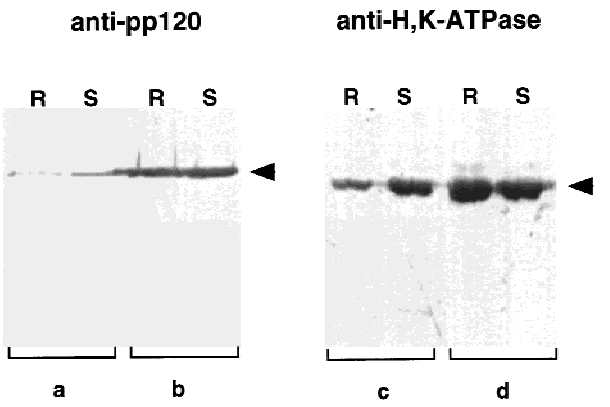

Fig. 3. Redistribution of pp120 from cytosol to the apical membrane with stimulation in association with the translocation of H,K-ATPase from tubulovesicles to the apical membrane. The apical membrane-rich fraction (*a, c*), cytosolic fraction (*b*), and microsomal fraction (*d*) from resting (*R*) or stimulated (*S*) glands (50 μg protein in each case) were separated on 7.5% SDS-PAGE and blotted on PVDF membranes. The blot was incubated either with anti-pp120 monoclonal antibody (1:10,000; left) or with anti-H,K-ATPase alpha subunit (1:25,000; right) and probed with HRP-anti-mouse IgG (1:5,000). The arrowhead shows the position of corresponding protein.

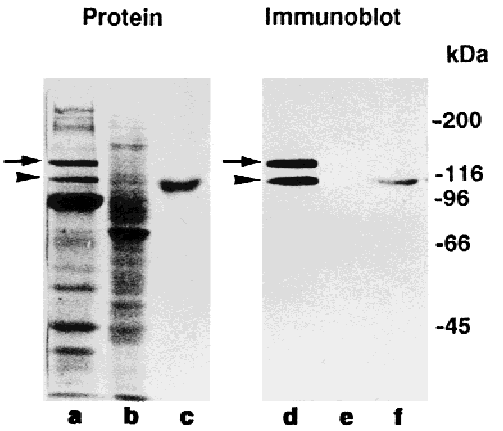

Fig. 4. Partially purified vinculin fraction obtained from gastric smooth muscle (*a, d*), apical membrane-rich fraction from stimulated gastric mucosa (*b, e*), and concentrated pp120 from cytosol of stimulated gastric mucosa (*c, f*), were separated on a 10% SDS-PAGE, transferred to nitrocellulose membrane, and stained with Amido Black (left, *a, c*). A duplicate sample was probed with anti-vinculin monoclonal antibody using 1:20 dilution, and visualized by HRP-conjugated anti-mouse IgG with 1:1000 dilution (right, *d, f*). The arrowhead indicates the position of vinculin, and the arrow indicates metavinculin. Note that the immunostained band in *f* migrates at a slightly higher position than the pp120 protein band in *c*, which migrates a little faster than 116K standard in this 10% gel.

panel. No reduction of pp120 in cytosol was visible, possibly because the total amount of pp120 attached to the apical membrane was so small compared with cytosolic pp120.

Distinction from Vinculin

It had been reported that the cytoskeletal protein, vinculin, was phosphorylated when parietal cells were stimulated (Cuppoletti & Malinowska, 1988). Since the molecular weight of vinculin is close to 120 kDa (Siliciano & Craig, 1987), we checked the possibility that pp120 is vinculin. Figure 4 shows Western blots, probed with monoclonal antibody against vinculin, for partially purified rabbit smooth muscle vinculin (lanes *a* and *d*), for the apical membrane-rich fraction from stimulated rabbit gastric mucosa (lanes *b* and *e*), and for protein concentrated from the 120 kDa region of cytosol from rabbit gastric mucosa (lanes *c* and *f*). In the vinculin control preparation, the anti-vinculin antibody recognized two protein bands with apparent molecular weights of 120 kDa (arrowhead) and 150 kDa (arrow), the former most likely representing vinculin and the latter metavinculin (Siliciano & Craig, 1987). No immunoreactivity was found in the apical membrane-rich fraction, while some vinculin (but not metavinculin) was found in the protein concentrated from the 120 kDa region of the cytosol. Close inspection of the cytosolic lanes revealed that the exact position of immunostaining was slightly lower than the main protein band, and the density of immunostaining was much less than expected from the amount of protein. The data suggest that pp120 is not vinculin, and that the 120 kDa region of gastric mucosal cytosol contains a small amount of vinculin. To confirm this, we extended the analysis to 2-D gels. The 120 kDa protein region form gastric cytosol was concentrated by preparative SDS-PAGE, and subjected to two dimensional electrophoresis (IEF/SDS-PAGE). As shown in Fig. 5, this fraction contained two proteins, pp120 (*c*) with a very low isoelectric point (about 4.5), and a slightly lower molecular weight protein, near neutrality (*b*). More than 90% of total radioactivity was attributed to the acidic pp120 (Fig. 5, upper panel). Further analysis was carried out by mapping peptide fragments of specific protein bands cut from gels (Fig. 5, left). As pointed out earlier, the peptide map of pp120 cut out from the gel of the apical membrane-rich fraction (*a*) showed an identical pattern to that from the cytosol (*c*). On the other hand, the neutral 120 kDa protein (*b*) was definitely different from the acidic pp120, and its mapping was indistinguishable from that of vinculin (*d*). Metavinculin (*e*) showed a pattern similar to vinculin, with some minor differences in the fragments below 30 kDa. It is clear from these results that pp120 is not vinculin.

Additional Features of pp120

To test the mode of association of pp120 with the membrane, the apical membrane-rich fraction was subjected

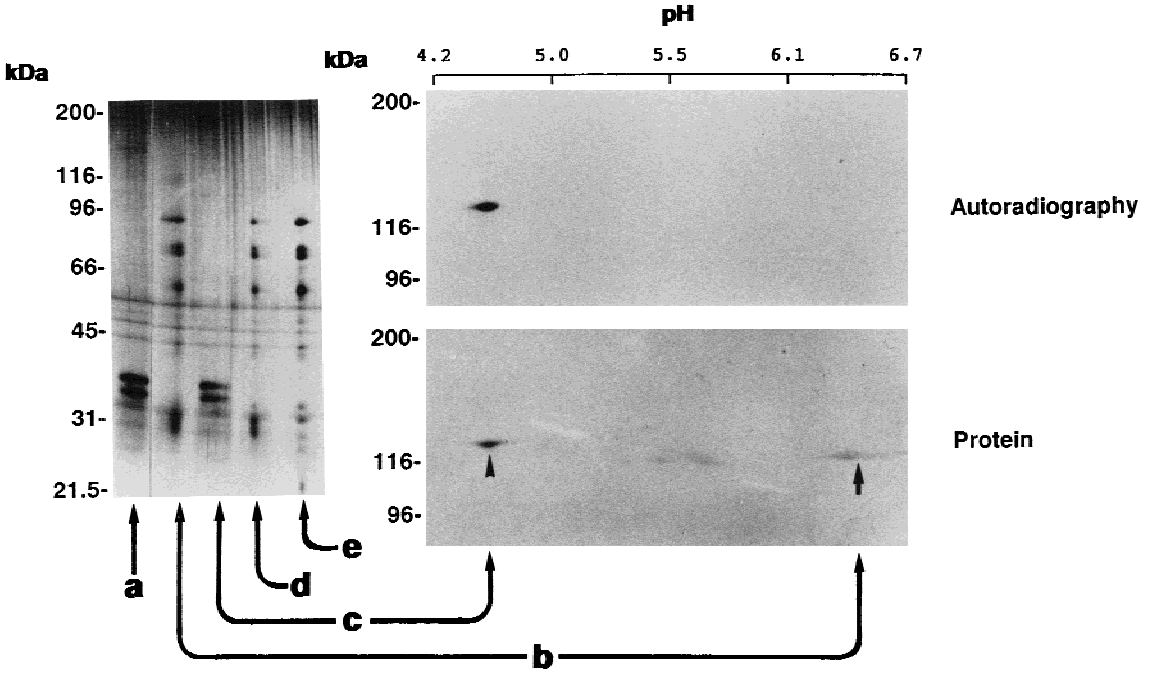

Fig. 5. Right: The 120 kDa region of the cytosolic fraction, prepared from ^{32}P-labeled, stimulated glands, was concentrated by preparative SDS-PAGE, and analyzed by two-dimensional electrophoresis (IEF/SDS-PAGE). The pH scale at the top was obtained by a surface electrode from an IEF tube gel run in parallel with the sample gel. The protein stained gel (lower panel) shows that pp120 (arrowhead) was focused around pH 4.5 with an apparent mass slightly larger than the 116 kDa marker; another protein, putative vinculin (arrow), focused around pH 6.5 with the exact position of 116 kDa. Note that virtually all of the radioactivity is attributed to the acidic protein, pp120 (upper panel). Left: Proteins cut out from a 2-D gel were analyzed by peptide mapping with V8 protease. (*a*) pp120 purified from the apical membrane-rich fraction by 2-D electrophoresis similar to the gel on the right; (*b*) putative vinculin spot cut out from the gel on the right (arrow); (*c*) pp120 spot from the gel on the right (arrowhead); (*d*) smooth muscle vinculin cut out from a gel as in Fig. 3*a;* (*e*) metavinculin obtained as in Fig. 3*d*.

to various treatments. The association of pp120 with the membrane was not disturbed by mechanical disruption or by interference with hydrogen bonding and/or ionic bonding. Thus pp120 was not released to the supernatant after hypotonic shock plus lyophilization and sonication (Fig. 6*a*), sonication in the presence of 50 mM Tris/1 mM EGTA/1 M Urea (Fig. 6*c*), or 50 mM EDTA, sodium borate (pH 9.0), sodium citrate (pH 4.5), 0.5 M NaCl, 0.1 M $MgCl_2$, 0.75 M guanidine isothiocyanate (*data not shown*). On the other hand, pp120 was completely solubilized by 1% NP-40/5 mM Tris/0.2 mM EDTA/300 mM sucrose (Fig. 6*b*), or 13 mM n-octylglucoside/5 mM Tris/0.2 mM EDTA/300 mM sucrose (Fig. 6*d*).

Phospho-amino acid analysis of ^{32}P-labeled pp120 revealed that phosphorylation certainly occurred on serine residues. Phosphorylation was not detectable on either threonine or tyrosine (*data not shown*).

Experiments Using Antibodies

Using either a monoclonal antibody or polyclonal antibodies for western blotting we established that pp120 migrates as a single band both in the cytosol and apical membrane-rich fraction. Screening of cytosolic fractions from other rabbit tissues with western blotting revealed that only the kidney contained pp120 at a detectable level, and this was much less than in fundic mucosa (Fig. 7). Occasionally a faint signal was detected from ileal tissue (*not shown*). When membrane fractions from various tissues were probed with antibody against pp120 a similar staining pattern was observed.

Isolated rabbit gastric glands were treated with cimetidine (resting) or with histamine plus IBMX (stimulated), fixed, probed with anti-pp120, and visualized by FITC-anti-IgG. From Figs. 8*A* and 8*B* it is obvious that pp120 is strongly expressed in the parietal cell, whereas no signal is present in chief cells or mucous neck cells. In the resting state (Fig. 8*A*) pp120 was distributed evenly throughout parietal cell cytosol except for the nuclear region. When the cells were stimulated (Fig. 8*B*), some aggregation of staining became apparent. The distribution of the aggregated stain would be consistent with a redistribution of some pp120 from cytosol to the apical secretory canalicular membrane in association with stimulation. When frozen sections of kidney were stained with anti-pp120, a faint but positive staining was observed in tubular cells, but absolutely negative in the glomerular region (*data not shown*).

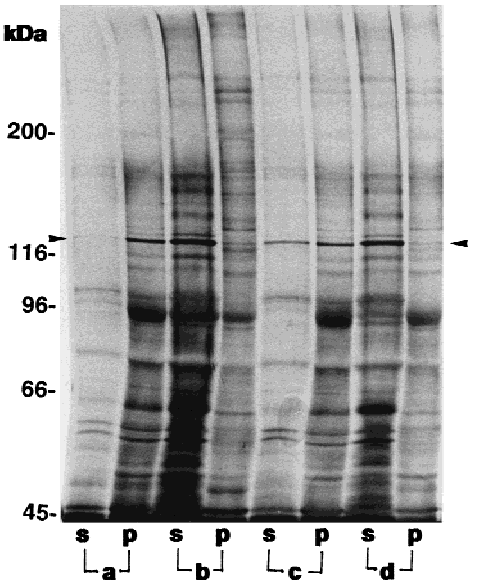

Fig. 6. Solubilization of pp120 from apical membrane-rich fraction. In sample *a*, about 75 μg of protein was suspended in 5 mM Tris/0.2 mM EDTA/300 mM sucrose, diluted 50 times with water, sonicated, lyophilized, reconstituted with water, and finally brought to 1 mg protein/ml. In other samples, the membranes were treated with 1% NP-40 (*b*), sonicated in the presence of 1 mM EGTA + 1 M urea (*c*), or treated with 13 mM n-octylglucoside (*d*). All samples were then centrifuged at 100,000 × *g* for 45 min. Supernatants (*S*) and pellets (*P*) were run on 6% SDS-PAGE and the gel was stained with silver. pp120 was specifically well stained (arrowhead) by this procedure. pp120 was resistant to sonication regardless of the presence of chelating agents and urea (*a* and *c*), while non-ionic detergents easily solubilized pp120 from the membrane (*b*, *d*). Note that NP-40 (*b*) solubilized both pp120 and 96 kDa peptide, H,K-ATPase, while octylglucoside. (*d*) solubilized pp120 but most H,K-ATPase remained in the membrane.

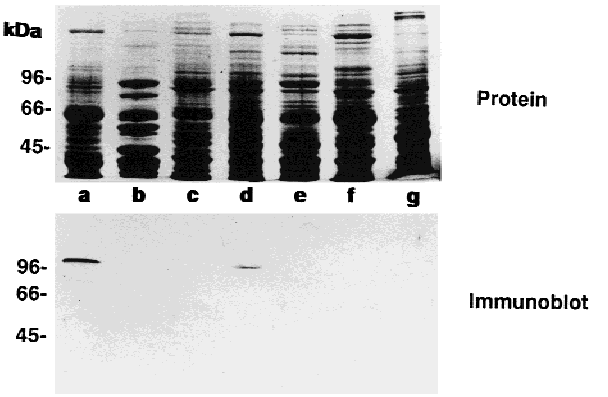

Fig. 7. Tissue distribution of pp120. Various rabbit tissues, including gastric mucosa (*a*), skeletal muscle (*b*), ileal mucosa (*c*), kidney (*d*), liver (*e*), lung (*f*), and whole brain (*g*), were homogenized and centrifuged at 100,000 × *g* for 45 min. The cytosolic fraction (supernatant), 50 μg for gastric mucosa and 100 μg for other tissues, were separated on 7.5% SDS-PAGE. The upper panel shows a gel stained with Coomassie Blue. The lower panel shows a western blot probed with anti-pp120 antiserum (1/500 dilution) and HRP-anti-rat IgG (1/5000 dilution).

In vitro Phosphorylation

Since we originally regarded pp120 as one of the substrates for cAMP-dependent protein kinase, we tested whether pp120 could be phosphorylated by protein kinase in vitro. However, our experiments demonstrated that phosphorylation of pp120 occurred independent of the presence of exogenous kinase. Figure 9 shows an example of in vitro phosphorylation. Partially purified pp120 from either the cytosol (DEAE-fraction, *A*) or apical membrane-rich fraction (*B*) was incubated with ^{32}P-ATP and 5 μg histone as a substrate, without any exogenous kinase, and analyzed by two-dimensional SDS-PAGE (6%/12%). pp120 was easily identified as a spot beneath the diagonal line (arrowheads). It is obvious from the figure that pp120 is the most heavily phosphorylated protein in both fractions. Among other proteins, whose phosphorylation was detectable as the exposure time was prolonged, exogenously added histone was preferentially phosphorylated (arrow). Although autophosphorylation of pp120 could be observed in the absence of exogenous substrate, it was increased by histone, polylysine, or protamine, but not by casein.

Using the DEAE-fraction, the effects of various agents on the phosphorylation of pp120 were examined and summarized in the Table. The kinase activity was dependent on Mg^{2+}, and it could be surrogated by Mn^{2+}. The activity was markedly augmented by the addition of histone, but was not affected by Ca^{2+} plus calmodulin. The inhibitor profile of the endogenous kinase activity did not fit that of cAMP-dependent protein kinase or protein kinase C, since 0.1 μM of heat-stable cAMP-dependent protein kinase inhibitor (about 30× greater than the Ki for cAMP-dependent protein kinase) and 10^{-4} M of H-7 (about 20× greater than the Ki for cAMP-dependent protein kinase and 30× greater than the Ki for protein kinase C) failed to inhibit the activity. The kinase did not seem to contain thiol groups in the active center because 10 mM N-ethylmaleimide, enough to abolish S6-kinase (Toru-Delbauffe et al., 1988) and a putative kinase involved in the KCl transport in erythrocytes (Gibson and Hall, 1995), showed no inhibition. In contrast, a general serine/threonine kinase inhibitor, quercetin (Srivastava and Chiasson, 1986) at 30 μM, markedly inhibited the phosphorylation of pp120.

The above data suggest that either pp120 is a highly suitable substrate for some other type of kinase, or that pp120 itself is a kinase. To test the latter possibility, we performed the following experiments. By using hydroxyapatite HPLC, almost pure pp120 could be collected in a nondenatured form. We suspect that some inactivation of pp120 may have occurred during the chromatography performed at room temperature, since total kinase activity was drastically decreased at this final

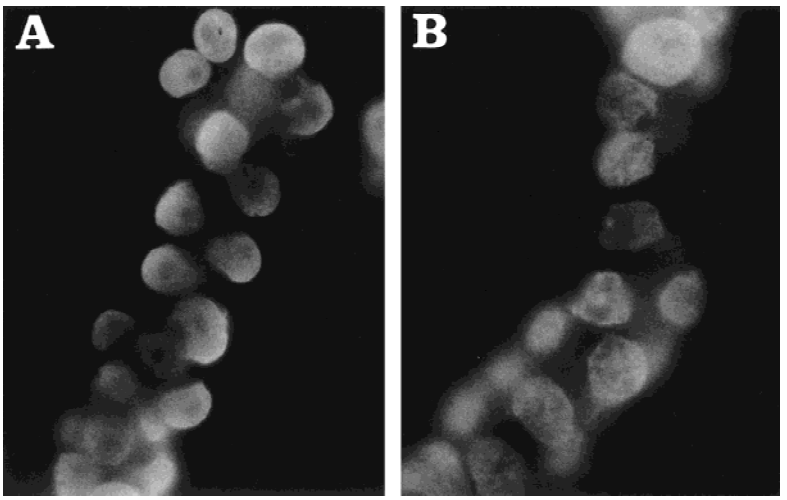

Fig. 8. Immunological localization of pp120 in rabbit isolated gastric glands. Resting (*A*) or stimulated (*B*) glands were fixed with formalin, and probed with anti-pp120 antiserum (1/50 dilution). The antibody was visualized by FITC-anti-rat IgG (1/100 dilution). In control glands probed with preimmune serum, fluorescence was undetectable (*not shown*).

step. Purified pp120 was then incubated with ^{32}P-ATP in the absence or presence of histone 1 or casein (Fig. 10). Autophosphorylation was difficult to detect in the absence of exogenous agents (lanes *a, d*); however, in the presence of either histone (lanes *b, e*) or casein (lanes *c, f*) autophosphorylation of pp120 was readily observed. It is also obvious from figure 10 that histone was more effective than casein.

Kinase assays were also performed in combination with immunoprecipitation. pp120, bound to Staphylococcus aureus Protein A with anti-pp120 monoclonal antibody, was incubated with ^{32}P-ATP in the presence of histone as substrates. Protein staining in Fig. 11 shows that the protein existed in this experiment was exclusively pp120 plus immunoglobulins. Under this condition, time-dependent phosphorylations of substrate and pp120 were clearly observed (Fig. 11). It was also noted that histone was more effective than protamine (*data not shown*).

Discussion

We have described the characteristics of a phosphoprotein, pp120, which may have a functional role in the secretory activity of the gastric parietal cell. Early studies identified pp120 along with another phosphoprotein, ezrin, in the apical membrane-rich fraction from stimulated parietal cells, and whose phosphorylations were closely correlated with the stimulation-dependent redistribution of the proton pump (Urushidani et al., 1987). With data from the present study, it became obvious that the phosphorylation of pp120 in the apical membrane-rich fraction was proportional to the amount of pp120 protein in that fraction, indicating that pp120 was translocated from another compartment in association with stimulation. The most likely origin was first thought to be the microsomal fraction, since it is well established that H,K-ATPase redistributes from the microsomal fraction to the apical membrane-rich fraction with stimulation. However, the microsomes, and any membrane fraction other than the apical membrane-rich fraction, had only a trace of pp120. On the other hand, the cytosol contained a considerable amount of the protein (Fig. 1), suggesting it might be the source for the stimulation-associated pp120 in the apical membrane-rich fraction.

Several experimental tests were used to demonstrate that the pp120-like protein in the cytosolic fraction is identical, or very similar, to pp120 in the apical membrane-rich fraction. For example, both proteins and their ^{32}P radioactivity comigrated on conventional two dimensional electrophoresis, they had similar peptide maps, both proteins showed the same sensitivity to silver staining, and antibodies recognized both species. Using two different concentrations of acrylamide in the first and second dimension of SDS-PAGE pp120 could be extracted as a single spot from other proteins. This anomalous migration in SDS-PAGE was a powerful tool for comparing pp120 in the cytosol and membrane fraction as well as demonstrating the similarity of phosphorylation pattern in vitro. Moreover, this technique, together with immunoblotting, confirmed that pp120 was accumulated in the apical membrane-rich fraction from stimulated cells and absent in the corresponding fraction from resting cells.

Contrary to the apical membrane-rich fraction, pp120 was present in the cytosol under all secretory conditions. Interestingly, the specific radioactivity was quite constant throughout the fractions, except for the resting apical membrane where quantification was practically impossible. This would suggest that cytosolic pp120 binds to the apical membrane without an apparent change in the state of phosphorylation when the cell is stimulated. The observation that there was no reduction of pp120 in the cytosol of the stimulated cell is not

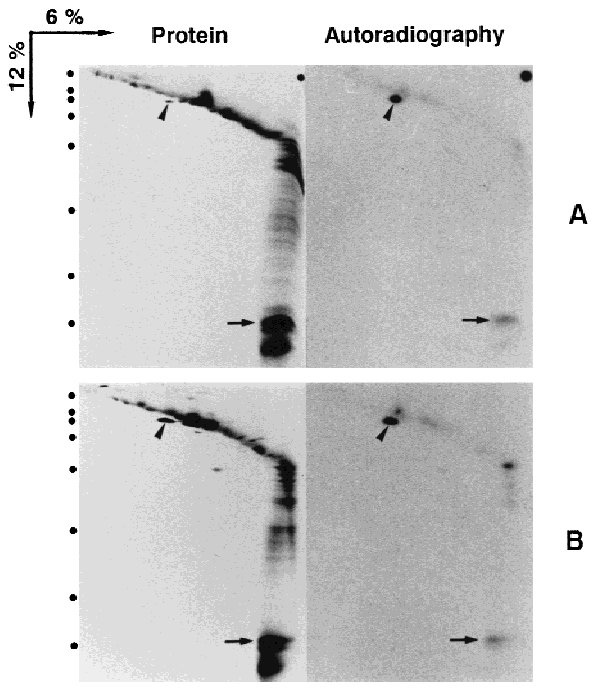

Fig. 9. In vitro phosphorylation of proteins from partially purified cytosolic pp120 (DEAE-fraction), (*A*) and apical membrane-rich fraction (*B*). (*A*) Partially purified cytosolic pp120 (DEAE-fraction) and (*B*) apical membrane-rich fraction were incubated with 0.2 mM [γ-^{32}P]ATP plus 1 mg/ml histone as an exogenous substrate. The reaction was terminated with SDS sample buffer and the samples were first separated on a 6% gel (horizontal), then each lane was cut out, and the gel strip was laid on top of a 12% gel (vertical). pp120 (arrowhead) was identified as a spot underneath the diagonal line. In the autoradiography some phosphorylation was observed in the histone band (arrow) as well as heavy phosphorylation in pp120. Since the amount of pp120 in *A* and *B* were quite different, different exposure times were employed to make the density of the autoradiographs equivalent. The gel was exposed for 5 days in *A*, while it was exposed 10 hr in *B*. The dots on the left of each panel indicate the position of molecular weight standards in the second dimension: 200, 116, 96, 66, 45, 31, 21.5, and 14.4, respectively, from the top to the bottom.

Table Effects of various agents on the phosphorylation of pp120 in the DEAE-purified fraction

Agents/Treatments	Phosphorylation of pp120 (% of control)	*N*
Mg^{2+} free (+EDTA 0.1 mM)	2.7 ± 1.5[b]	4
Mn^{2+} 1 mM (instead of Mg^{2+})	124 ± 9.0	2
Histone 0.1 mg/ml	547 ± 158[b]	5
Ca^{2+} 0.1 mM + calmodulin 1000 U/ml	89.0 ± 6.2	3
PKA inhibitor[a] 0.1 μM	91.1 ± 5.2	4
H7 0.1 mM	97.0 ± 2.0	3
N-ethylmaleimide 10 mM	96.9 ± 2.1	4
Quercetin 30 μM	9.5 ± 4.7[b]	3

The DEAE-purified samples were brought to 50 mM PIPES, pH 6.8, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM PMSF, 10 μM pepstatin, 0.2 mM [γ-^{32}P]ATP, and mixed with test material. The mixture was incubated at 25°C for 15 min and the sample was then analyzed by SDS-PAGE and autoradiography. The band corresponding to pp120 was scanned for quantification, and expressed as % of control.
[a]Heat stable cyclic AMP-dependent protein kinase inhibitor peptide.
[b]Statistically significant from control at $P < 0.05$ by paried *t*-test.

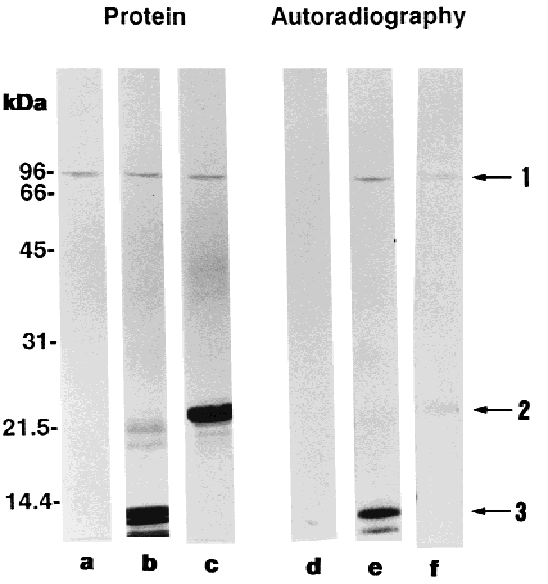

Fig. 10. Protein kinase activity of HPLC-purified pp120 fraction. Phosphorylation from [γ-^{32}P]ATP was performed without (*a*, *d*) or with exogenous substrates, histone (*b*, *e*) or casein (*c*, *f*). Phosphorylation of pp120 (arrow 1) was undetectable in the absence of exogenous substrate (*d*), while added substrates were phosphorylated as well as stimulating the phosphorylation of pp120. The stimulation was more prominent by histone (arrow 3) than by casein (arrow 2). Note that HPLC-purified pp120 runs as almost a single band (*a*). Because of the nature of the gel (7.5%-15% gradient), the apparent molecular weight of pp120 is as low as 96 kDa.

contradictory to the translocation hypothesis. The apical membrane-rich fraction contains about 3% of the total glandular protein, while the cytosolic fraction contains 30% or more (Urushidani & Forte, 1987). Based on gel staining the amount of pp120 per mg of apical membrane fraction protein was less than half of the pp120 per mg cytosolic protein. Thus we estimate that the membrane-bound form of pp120 was less than 5% of the soluble form, even in the maximally stimulated state, suggesting that the reduction in the soluble form associated might not be detectable when translocation to the membrane-bound form occurred.

The translocation of pp120 was also supported by the immunohistological examination. In the resting state, pp120 existed evenly in the cytosol of the parietal cells. When cells were stimulated, there was a faint staining in the pattern analogous to the apical secretory canaliculi of parietal cells. This stimulus-dependent change was similar to, but less prominent than, that observed in the staining by anti-H,K-ATPase (Hanzel et al.,

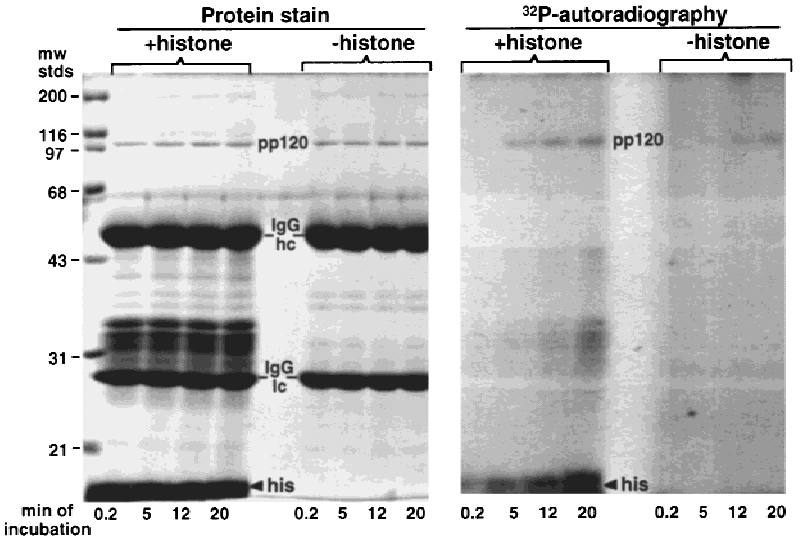

Fig. 11. Protein kinase activity of immunoprecipitated pp120. The cytosolic fraction from rabbit gastric homogenate was treated with a monoclonal antibody against pp120, and the antigen/antibody complex was adsorbed with *Staphylococcus aureus* protein A. The precipitated material was washed and added to the protein kinase mixture, with or without 1 mg/ml histone (hist) as an acceptor protein. Samples were incubated at 37°C for various times, as shown, and developed on 10% SDS-PAGE for protein staining and ^{32}P-autoradiography. When histone was present both the acceptor histone and pp120 were progressively phosphorylated over the 20 min of incubation. In the absence of histone phosphorylation of pp120 was markedly reduced. Positions of the heavy (IgG hc) and light (IgG lc) chains of the immunoprecipitating antibody are shown.

1989). This might reflect the fact that the translocation of H,K-ATPase is rather complete, while that of pp120 is partial.

The next issue is the elucidation of the physiological role of pp120. Using antibodies, it was revealed that pp120 exists almost exclusively in the parietal cell, with little to no signal being detected in other gastrointestinal epithelia. This suggests that pp120 does not serve a common physiological function in epithelial cells of the digestive tract, in contrast to other phosphoproteins in the apical membrane, e.g., 80K/ezrin, which is a major component of brush border membranes (Hanzel et al., 1989, 1991). The existence of pp120 in tubular cells in the kidney, although the amount is small, is suggestive, since this cell type, which also secretes acid, is reported to show some rearrangement of apical and basolateral membranes (Al-Awqati, 1996).

Vinculin is a 120 kDa protein which was reported to be phosphorylated when parietal cells were stimulated with histamine (Cuppoletti & Malinowska, 1988). Our experiments demonstrate that the apical membrane fraction does not contain vinculin, and that vinculin in the cytosol is a minor component with respect to the phosphorylation as compared to pp120. This does not totally exclude the possibility that vinculin binds to the apical membrane with stimulation, or that vinculin might be involved in parietal cell function. The main point here is that pp120 is not vinculin.

Several features of pp120 remind us of a neuron-specific protein, B-50/GAP-43 (for review, Oestreicher et al., 1997). This phosphoprotein, although its physiologic role is still unknown, has been studied extensively in relation to nerve growth or synaptic plasticity. GAP-43, with an isoelectric point of 4.5, close to that of pp120, also shows anomalous mobility in SDS-PAGE (Benowitz & Routtenberg, 1987; Jacobson, Virag & Skene, 1986). The apparent molecular weight of GAP-43 was 65 kDa in 6% PAGE, and decreased as low as 40 kDa when acrylamide was increased to 12%. More intriguing, the true molecular weight calculated from the amino acid sequence is only 24 kDa, and the product obtained from in vitro translation shows the same anomalous mobility in SDS-PAGE, excluding the possibility of the post-translational modification. The anomalous mobility is thus thought to be the low binding of SDS to GAP-43 because of its high negative charge (Benowitz & Routtenberg, 1987), and the same mechanism might apply to pp120, with the implication that the true molecular weight of pp120 might be much smaller than 100 kDa.

It is important to analyze the mode of binding to membranes when the physiologic role of pp120 is considered. Although there is no definitive evidence that the membrane bound form of pp120 is exactly the same as the soluble form, any drastic modification in structure would not be expected in view of the constancy in two dimensional electrophoresis and peptide mapping. The existence of a predominant soluble form excludes the possibility that pp120 is an integral membrane protein, while it is also difficult to regard this protein as a peripheral membrane protein in view of its resistance to treatments such as changes in ionic strength or pH, sonication, chelating of divalent cations, and urea. Our early experiments showed that 80K/ezrin also showed a similar resistance to the above treatments (Urushidani et al., 1989). Ezrin was also resistant to non-ionic detergent at low ionic strength, and subsequently recovered in the cytoskeletal fraction, suggesting the importance of its association with F-actin (Urushidani et al., 1989; Hanzel et al., 1991). On the other hand, pp120 was easily solubilized by non-ionic detergent. With as little as 13 mM

n-octylglucoside, pp120 was perfectly solubilized while H,K-ATPase, a typical integral protein, remained in the membrane fraction. Under this condition, H,K-ATPase activity was not inhibited but rather activated more than two-fold (Urushidani & Forte, 1987). This at least excludes the possibility that pp120 is an activating factor for H,K-ATPase.

There are other unusual cases where the mode of protein-membrane association is unclear. GAP-43 is known to exist as a synaptosomal membrane-bound form, although no consensus hydrophobic domain is present in the primary sequence to explain the membrane binding ability (Benowitz & Routtenberg, 1987). Furthermore, similar to features of pp120, GAP-43 cannot be solubilized by the change of ionic strength or chelation of divalent cations, but is easily solubilized by low concentration of non-ionic detergent (Aloyo, Zwiers & Gipsen, 1983). Although the palmitoylation of both Cys 3 and 4 at the N-terminus of GAP-43 has been attributed to its membrane association (Skene & Virag, 1989), a putative membrane-located anchoring protein has also been suggested (De Graan et al., 1993).

The most interesting feature of pp120 is that it seems to be closely related to a new type of protein kinase. It is unlikely that its phosphorylation is regulated by PKA because pp120 is highly phosphorylated in the resting state. Two completely different fractions, partially purified cytosolic fraction and apical membrane-rich fraction, showed a strikingly similar mode of phosphorylation, i.e., high specific activity phosphorylation of pp120 and the preferential phosphorylation of histone. These data suggest that pp120 is itself a protein kinase, or that it is a highly suitable substrate very closely associated with a protein kinase. These possibilities were further supported by the experiments using HPLC-purified pp120, or immunoprecipitated pp120, although it is difficult to distinguish between them. On the one hand, as pp120 was purified no protein bands other than pp120 were visible on SDS-PAGE suggesting that pp120 itself is a kinase. On the other hand, the protein kinase activity of the pp120 enriched samples tended to decrease with the severity of the purification procedure consistent with the possibility that pp120 is not a kinase but tightly associated with, perhaps even an activator for, a protein kinase. This latter possibility resembles the case of GAP-43 (Aloyo et al., 1983), which is cosolubilized and copurified with C-kinase from the membrane. However, antagonist work clearly showed that the pp120-associated kinase is not C-kinase. Moreover, from preliminary tests presented here it appears that pp120-associated kinase activity is also different from that of cAMP-dependent kinase or calmodulin-dependent kinase. The general characteristics of the pp120 associated kinase appear more similar to the reported polypeptide-activated protein kinase (Abdel-Ghany, Riegler & Racker, 1984) although based on the rate of autophosphorylation and molecular weight they could not be the same molecule.

In conclusion, pp120 is closely associated with a new type of kinase, almost specifically expressed in the gastric acid secreting cell, where it exists as a soluble form in a resting state, and translocates from cytosol to the apical membrane when the parietal cell is stimulated. Further analysis of this interesting protein is now in progress in our laboratory.

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