Identification of the Major Site of Rat Prolactin Phosphorylation as Serine 177*

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Phosphorylation of prolactin by endogenous protein kinases within isolated secretory granules was shown to result in the production of both phosphoserine and phosphothreonine residues. The majority of the radio-label was determined to be present in the C terminus of the molecule after specific cleavage with glandular kallikrein. Glandular kallikrein cleaves in three places at the C terminus, liberating three small peptides, only one of which contains a phosphorylatable residue. Sequencing of this phosphopeptide showed it to be Arg-Thr-Arg-Leu-Ser-Asp. Thus the major site of prolactin phosphorylation was determined to be serine 177. Using a synthetic peptide equivalent to this region of the molecule (Ser161-Val180), serine 177 was demonstrated to be a substrate for protein kinase A as well as for one of the endogenous granule kinases. Inclusion of the synthetic peptide in an endogenous granule phosphorylation reaction resulted in competition for the kinase and reduced phosphorylation of prolactin. Protein kinase A phosphorylation of purified prolactin resulted in the production of only phosphoryserine and primarily the most abundant (monophosphorylated) variant. We conclude that serine 177 is the major in vivo phosphorylation site of rat prolactin and that phosphorylation of this site can be reproduced by protein kinase A in vitro. The minor threonine phosphorylation site was demonstrated by two-dimensional tryptic peptide mapping and mass analysis to be either threonine 58 or 63, both of which are contained within a single peptide.

For many years prolactin (PRL)† was considered an unmodified polypeptide hormone. It is now clear, however, that post-translational processing of PRL causes it to be phosphorylated (e.g. Refs. 1 and 2), glycosylated (e.g. Ref. 3), and variously proteolytically cleaved (e.g. Refs. 4–6). The phosphorylation of PRL within pituitary cells has been demonstrated to occur in vivo in the rat (1), chicken (7), and cow (2). Phosphate analysis of purified preparations of PRLs from different species showed them to be variously phosphorylated with molar ratios of hormone to phosphate of 1.0:0.2 for ovine and rat and 1.0:0.7 for turkey (7).

Functional studies from this laboratory have determined that monophosphorylated PRL is an antagonist to non-phosphorylated PRL in two cell systems where non-phosphorylated PRL promotes cell proliferation (8, 9). It is therefore important to establish the sites of PRL phosphorylation so that these may be reproduced in vitro for further analysis of this antagonism which operates through a single receptor (9, 10).

In our earlier studies, we used a variety of purified protein kinases in an attempt to identify potential phosphorylation sites (1). This approach, however, while illustrating that PRL is a very readily phosphorylated molecule, did not narrow the search because such a variety of protein kinases with very different consensus recognition sequences were found capable of phosphorylating PRL. For PRL from other species, only protein kinase A (PKA) has been tried and shown to successfully phosphorylate ovine, chicken, and turkey PRL (7).

In this article we present evidence that PRL is phosphorylated on both a serine and threonine residue and that only the serine phosphorylation, which is the major site, can be reproduced by PKA.

MATERIALS AND METHODS

Secretory Granule Preparation and Characterization—PRL secretory granules were isolated from female rat anterior pituitaries as described by Zanini and Giannattasio (11) and modified by Greenan et al. (12). This procedure results in a highly enriched fraction of large, dense PRL granules (11, 12). Three to five μl (4–6 μg) of granules (suspension in 0.32 M sucrose) were subjected to SDS electrophoresis under reducing conditions on a 10% gel (30 mA, 3.5 h). Separated proteins were stained with Coomassie Blue and the amount of protein present in the PRL band determined by laser densitometry. The PRL band was identified by (a) blotting a duplicate lane onto nitrocellulose, probing with rabbit anti-PRL, and development of a peroxidase-conjugated second antibody according to previously published methods (13), and (b) by reference to co-run and stained molecular mass markers.

Endogenous Phosphorylation of PRL in Secretory Granules—Fractionated secretory granules were sonicated on ice for 10 s at position 4 using a Sonifier Cell Disrupter (Heat Systems-Ultrasonics, Plainview, N. J.). Typically 40 μg of secretory granules were incubated with 250 μCi of [γ-32P]ATP (4500 Ci/mmol, ICN, Irvine, CA) in 60 mM Tris-HCl, pH 7.4, in a total of 80 μl at 37 °C for 3 h. 6 μl β-Glycerophosphate was included during this 6-h incubation to reduce phosphatase activity. For analysis on gels, the reaction was terminated by boiling for 3 min with an equal volume of 2 × sample buffer (to give 6.2 μl Tris, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, pH 6.8). Electrophoresis in 10% gels was followed by Coomassie Blue staining and either exposure to autoradiographic film after drying or in the wet state (with 10% methanol to prevent cracking) if further processing was to take place. Film used was Kodak X-Omat (Kodak, Rochester, NY) and exposure was at −70 °C.

Phosphoamino Acid Analysis and Phosphopeptide Mapping of Endogenously Labeled Intragranular PRL—After phosphorylation by endogenous granule kinases and separation on a 10% gel, the phosphorylated PRL band was excised and sliced into 1-mm cubed pieces. These were rinsed 6 times (10 min each) in double distilled deionized water to remove SDS and once in 50 mM NH₄OH, pH 8.0, for 3 min. The PRL band was excised and sliced into 1-mm cubed pieces. These were incubated with L-Tosylamido-2-phenylethyl chloromethyl ketone-treated sequencing grade trypsin (Promega, Madison, WI) was used to digest the PRL (1:10, w/w) in 200–250 μl of 50 mM NH₄OH, pH 8.0, at 37 °C for 20–40 h. Five μl of glacial acetic acid was then added to terminate the reaction. The gel suspension was then separated into component parts by passage through a 10-KDa filtration membrane (Ultrafree-MC, Millipore, Bed-

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The abbreviations used are: PRL, prolactin; PKA, protein kinase A; aa, amino acid(s); HPLC, high performance liquid chromatography; rf, ratio to front.
Peptides below 10 kDa (undigested PRL is 24 kDa) were lyophilized and dissolved in either 10 μl of electrophoresis buffer (pyridine-acetic acid:H2O at 10:100:1890, pH 3.5) for peptide mapping or 0.5 ml of 6% sequencing grade HCl (Pierce) for phosphoamino acid analysis.

For two-dimensional phosphopeptide mapping, horizontal electrophoresis was for 2 h at 450 V with cooling water at 6°C on a 20 × 20-cm silica gel plate (EM Separations, Gibbstown, NJ). This was followed by chromatography (butanol-pyridine-acetic acid:H2O at 60:40:12:48) until the solvent was 2 cm from the top of the plate, according to the procedure of Boyle et al. (14). The position of free phosphate was determined by duplicate runs of 3 × 107 cpm [32P]H3PO4 (ICN). Plates were allowed to dry and were then exposed to film at −70°C.

Samples for phosphoamino acid analysis, produced by trypsinization (above), were transferred into vacuum hydrolysis tubes for hydrolysis at 110°C in a heating block for 1 h. Hydrolysates were dried and mixed with standards (2 μg each of phosphotyrosine, phosphoserine, and phosphothreonine) (Sigma) in water and applied to a 20 × 20-cm silica gel plate. Electrophoresis was for 4 h at 400 V with cooling water at 6°C. Plates were air-dried, sprayed with ninhydrin (0.1% in butanol) to identify the location of the standards, and then exposed to film at −70°C.

For phosphopeptide mapping, a constant volume of each sample is separated by electrophoresis, and the gel is fixed, stained, and destained. The gel is then sliced into pieces, and each piece is extracted with acid and then with organic solvents. The eluates are combined and subjected to microsequencing.

**Fig. 1. Sequence of rat PRL.** Sequence taken from Cooke et al. (31). Predicted tryptic cleavage sites are shown by small arrows. Kallikrein cleavage sites are shown by large arrows as per Powers and Hatala (19).
Intramembranous Phosphorylation of PRL—Phosphorylation of PRL by the endogenous kinases of secretory granules is illustrated in Fig. 3A. Under these conditions about 30% of the PRL is phosphorylated, as assessed by densitometric analysis of the phosphoprotein on two-dimensional protein gels. Besides PRL, which is a major phosphoprotein, a phosphoprotein at approximately 21 kDa, which is most likely a previously characterized cleaved fragment of PRL produced by the granular kallikrein (17), is seen (see later for further analysis). This 21-kDa protein is produced in granules which have been incubated at 37 °C and hence it is not present in unincubated granules such as those illustrated in Fig. 2. The other phosphoproteins may represent the protein kinases and/or other very highly phosphorylated minor proteins of the granules.

Phosphoamino acid analysis of the excised, endogenously phosphorylated PRL band showed phosphorylation on both serine and threonine residues (Fig. 3B). No phosphotyrosine was detected. The amount of phosphothreonine was larger than phosphoserine, but this is likely due to the greater lability of phosphoserine. As will be shown below, the major site of PRL phosphorylation was on a serine.

Tryptic phosphopeptide mapping of endogenously phosphorylated PRL showed four labeled spots/areas. By a variety of techniques, one spot was identified as free phosphate, two as distinct peptides and one, at the origin, as poorly digested, C-terminal regions of PRL. The vast majority of the incorporated label remained with the poorly digested C-terminal region (Fig. 3C).

Serine 177 Phosphorylation Site—To overcome the major problem of incomplete trypsin digestion, which occurred with or without alkylation of the cysteines, and because consensus sequence analysis suggested that one phosphorylation site was close to the C terminus, we took advantage of the well defined C-terminal cleavage of PRL by glandular kallikrein and analyzed the peptide products and remnant PRL on high percentage Tris glycine gels. Glandular kallikrein cleaves at 3 sites at the C terminus of PRL (Fig. 1) resulting in the production of an undecapeptide, RDSKVDNYLK (amino acids 175–185), a tripeptide FLR (amino acids 186–188), a nonapeptide, COIVH-KNNC, and the remaining approximately 21-kDa remnant portion of the PRL molecule (19). An intermediate band of 23 kDa represents loss of only the nonapeptide. The only potential site for serine or threonine phosphorylation within this region is present in the undecapeptide at serine 177. Following intragranular phosphorylation by endogenous kinases, digestion with exogenous kallikrein was initiated. Kallikrein is a natural component of PRL secretory granules (17) and so some cleavage was expected in the samples to which exogenous kallikrein was not added as soon as the requirement for β-mercaptoethanol and Triton X-100 was fulfilled in the experimental protocol (necessary for efficient kallikrein cleavage of PRL) (19). Electrophoresis and autoradiographic analysis of this control (Fig. 4, lane 1) therefore indicated a band with an r f value equivalent to the undecapeptide, which for the sake of simplicity we hence refer to as the kallikrein peptide (KK) peptide. The tripeptide is not retained on the gel and the nonapeptide, which has a different r f value, has no site for phosphorylation. It is clear from Fig. 4, however, that the addition of exogenous kallikrein increased the production of the radiolabeled KK peptide, which travels with an r f value of 0.62. The average increase from four experiments was 35.6 ± 3%. Concomitant with an increase in label in KKp was a loss of label in the PRL band. As judged by analysis of lane 2, by far the greatest amount of radioactivity was present in the peptide and not in the remainder of the molecule (21-kDa radiolabeled band). This identifies the major site of PRL phosphorylation as serine 177 and the minor site as N-terminal to amino acid 175. HPLC separation of the individual peptides liberated by kallikrein confirmed the presence of only one completely cleaved labeled peptide. Sequencing of this peptide showed it to be RDSKVDNYLK (Table I).

To further analyze phosphorylation at the C-terminal site, a 20-amino acid peptide equivalent to amino acids 161–180 of PRL was synthesized. This sequence, SKDLAFYNNIRCLRRDKSHV, was chosen so as to include the Arg six places and Phe 11 places to the N terminus of the serine because equivalent residues in the PKA inhibitor protein have a large positive effect on affinity for PKA (20). When included in the endogenous granule phosphorylation reaction, this peptide became phosphorylated (Fig. 5A). This is illustrated in lanes 1, 2, and 3 where 0, 18, or 36 μg of the peptide were added, respectively. Lane 1 shows no labeled 20-aa peptide and lanes 2 and 3, increasing amounts of the 20-aa peptide. The 20-aa peptide runs slower (r f value of 0.60) than the KKp, produced by activity of the granular, endogenous kallikrein. When the amount of radiolabeled intact PRL in lanes 1, 2, and 3 was quantified by densitometry (Fig. 5B), it was also clear that the added peptide competed with intact PRL for phosphorylation by a granular
kinase. If a synthetic peptide containing the putative threonine phosphorylation site (Leu61-Glu68) was included (lane 4), no phosphorylation of it was seen. This is discussed in detail below.

In Vitro PKA Phosphorylation of Purified PRL and the 20-aa C-terminal Peptide—Despite the likelihood, based on consensus sequence analysis, that serine 177 was a site for PKA phosphorylation we wished to determine this unequivocally. As demonstrated in Fig. 6, the 20-aa peptide representing amino acids 161–180 of PRL competes with the provided PepTag substrate in the commercial PKA assay kit. With limiting enzyme, 24 μg of the PRL peptide inhibited phosphorylation of 4 μg of PepTag substrate by ~50%. On a molar basis this is translated to 2 mol of PRL peptide competing equally with 1 mol of PepTag. This demonstrates that although the Arg six places and Phe 11 places N-terminal to serine 177 in the PRL peptidemay increase affinity for PKA, the Asp in the X position of the recognition sequence, RRXS, in PRL must be less than ideal. Otherwise, one would have predicted that the PRL peptide would have been a much better substrate than the PepTag. Nevertheless, it is clear that this region of PRL is an excellent substrate for PKA. Other non-phosphorylatable peptides and phosphorylatable peptides without a PKA consensus sequence had no effect on PepTag phosphorylation.

In vitro phosphorylation of purified PRL (NIDDK I5) by the catalytic subunit of PKA is shown in Fig. 7A. The degree of PRL phosphorylation is dependent on the amount of PKA catalytic subunit used. Under the conditions used, a maximum of about 18% of the PRL is phosphorylated, as assessed by incorporated moles of phosphate or densitometric analysis of the phospho-protein on a two-dimensional protein gel. Phosphoamino acid analysis of PKA-phosphorylated PRL showed only the presence of phosphoserine (Fig. 7B). Two-dimensional peptide maps were the same as endogenously phosphorylated PRL (showing

| Residue | Amino acid | pmol (raw) |
|---------|------------|------------|
| 1       | Arg        | 70.5       |
| 2       | Asp        | 36.7       |
| 3       | —*         | —*         |
| 4       | His        | 13.0       |
| 5       | Lys        | 16.6       |
| 6       | Val        | 19.0       |
| 7       | Asp        | 16.8       |
| 8       | Asn        | 14.5       |
| 9       | Tyr        | 13.6       |

* Position of serine 177. Phosphoserine is lost during sequencing so that this position was not assigned by the computer. Main amino acids identified in this cycle were dehydroalanine, 7.5 pmol; Leu, 9.6 pmol. Leucine background was about at this level in all cycles.
a highly-labeled, poorly-digested spot at the origin) except that one of the two distinct peptide spots (later identified as the threonine peptide) was missing (not shown). HPLC analysis following extended trypsin cleavage (40 h) showed the majority (90%) of the incorporated radiolabel to be present in the flow-through of a reverse phase C18 column. (Free ATP was removed with AG-X1 resin (21) and its removal was checked by thin layer chromatography.) This was anticipated should phosphorylation mainly occur at serine 177. Deduced from the sequences of predicted tryptic peptides (Fig. 1) (cut sites determined by software of Genetics Computer Group, Madison, WI), peptides in the region 172–179 would be expected to pass through a C18 column. To test behavior on a C18 column, a peptide corresponding to a less-than-ideal digestion of this region, constituting amino acids 173–180 (LRRDSHKV), was synthesized. The cysteine at position 172 (normally present in the tryptic peptide) was not included in order to prevent potential problems with dimerization. The addition of a valine at the C terminus restored approximate size and charge balance. Even when not phosphorylated and even though larger than some potential peptides in this region, this peptide passes through a C18 column (data not shown). Thus, on the basis of incorporated counts most PKA phosphorylation was at serine 177.

Two-dimensional Gel Analysis of PKA in Vitro Phosphorylated PRL—Two-dimensional gel analysis of PKA-phosphorylated purified PRL (Fig. 8, A and B) showed radiolabeled phosphate incorporation predominantly into the monophosphorylated variant. Since purified PRL already contains unlabeled mono- and diphosphorylated PRL as well as non-phosphorylated PRL and phosphorylation increases the charge on the PRL, this indicates that the phosphorylation site is available in non-phosphorylated PRL, but not in mono- or diphosphorylated PRL (see "Discussion" for a more detailed explanation). It is therefore the first and major phosphorylation.

Preliminary Analysis of the Minor, Threonine Phosphorylation Site—Evidence thus far presented has confirmed that the major site for phosphorylation of PRL in vivo is serine 177. In addition, we know that in vivo phosphorylated PRL also contains phosphothreonine. Two-dimensional phosphotryptic peptide mapping of endogenously labeled PRL showed a phosphopeptide not present in the PKA phosphorylated material. Since the amount of this peptide was limiting, only mass analysis was carried out. The results are shown in Fig. 9. The mass of 1708 obtained can only be ascribed to the tryptic peptide Ala52-Lys72, having the sequence AINDCPTSSLATPEDK. No other peptide, even allowing for incomplete tryptic digestion and any known artifacts of tryptic digestion or subsequent processing, have a comparable mass. The H^+ peptide has a mass of 1662. β elimination of cysteine, which occurs very readily in alkaline conditions (22), reduces this to 1628. This particular preparation of PRL was not alkylated and trypsin digestion occurs in an alkaline environment. Phosphorylation of one residue in the sequence raises the mass to 1708. Since (a) there are no threonine residues in the poorly digested C-terminal region of PRL, (b) this phosphopeptide is only present in the PRL phosphorylated by endogenous kinases, and (c) only endogenously phosphorylated PRL contains phosphothreonine, it is likely that the phosphorylation site is threonine 58 or 63 rather than serine 59 or 60.
Further analysis of this threonine phosphorylation site is complicated by the presence of two threonines within a single peptide. On the basis of consensus sequence analysis for known protein kinases, the most likely phosphorylation site was judged to be threonine 63 and a peptide representing amino acids 61–68 (Tp) was included in an endogenous granule phosphorylation reaction, illustrated previously in Fig. 5 (lane 4). No phosphorylation of this very small peptide was observed. The threonine-containing peptide (Tp), as determined from the silver-stained gel, ran slower than the kallikrein peptide and faster than the 20-amino acid peptide and contained no detectable radioactivity.

Specificity of PKA Phosphorylation of Purified PRL—Besides the peptides around serine 177, all other serine and threonine-containing PRL tryptic peptides should be retained on a C18 column and hence HPLC analysis of tryptic digests of the in vitro PKA-phosphorylated material was a good test for additional and, from our point of view, undesirable phosphorylation sites.

One radiolabeled peptide was found representing 10% of incorporated radioactivity. Sequencing established this peptide as IISQAYPEAK (amino acids 131–140). When a synthetic peptide, EKIISQAY (amino acids 129–136 of PRL), containing
After a typical in vitro granule reaction, however, was very different. The quantities of each product were found to be much higher than in the absence of PRL on two-dimensional gels (12). The quantities of each product produced, in granule reactions, however, was very different. The apparent mono- phosphorlylated variant represented 30–40% of total PRL and the diphosphorylated variant between 1 and 5% (12). In experiments where radiolabeling was not used, the production of the phosphorylated variants did not depend on the addition of exogenous ATP and, although reduced, phosphorylation was not eliminated by the removal of granule membranes (12). Granule membranes have been shown in another species to possess a proton pump (23). Since ATP does not have to be provided and phosphorylation occurs in the absence of an ATP-generating pump, we deduce that there is a store of ATP within the granules and assume that this is one reason why we have not been able to radiolabel granule PRL with endogenous kinases to very high specific activity. Nevertheless, we have been sufficiently successful in unequivocally identifying the major site of PRL phosphorylation. Analysis of the major site of PRL phosphorylation did not yield to traditional approaches because of the inefficient cleavage of the C terminus of PRL by trypsin. Twenty hours of digestion left very large peptides which stayed at the origin when subjected to two-dimensional peptide mapping. Even with complete digestion, however, one would not be able to catch the phosphorylated peptide by traditional means because it runs with free phosphate on a two-dimensional peptide map and straight through a C18 column. It is for this reason that we turned to kallikrein digestion of the molecule and analysis on high percentage gels. This approach had the significant advantage of producing fewer peptides and, from previous work from Powers’ (17) group using purified PRL (19) and our group using PRL secretory granules, we knew the correct conditions for efficient digestion. These conditions include reduction of disulfide bonds and treatment with Triton X-100 to access the C terminus of PRL, indicating the relative unavailability of this region of the molecule.

Based on the results of kallikrein digestion of intragranular endogenously phosphorylated PRL, by far the largest amount of radioactive phosphate is present in the C terminus of PRL in the peptide RD5KHVKDNYLK (175–185). Since the kallikrein digestion experiments involved the least processing and no exposure to either alkali or acid, this is strong evidence that serine 177 is the major site of PRL phosphorylation. To confirm that this region of the molecule was phosphorylated by the endogenous granule kinases, the 20-amino acid peptide representing amino acids 161–180 of PRL (SKDLAYNNRCLRDRSHKV) was included during radiolabeling reactions. Not only did this peptide become radiolabeled, but it prevented phosphorylation of some intact PRL in the granules. This competition demonstrates that the peptide was truly phosphorylated by PRL kinase and not by a protein kinase on the cytoplasmic (outside membrane) surface of the granules or by a contaminant of the granule fraction. Serine 177 is a highly conserved residue among PRLs from different species (24). This region of the molecule has been determined to be critical for biological activity (25). Our previous studies have demonstrated that monophosphorylated PRL is an antagonist to non-phosphorylated PRL. Thus one might have predicted that this would be an important site for phosphorylation.

Based on consensus sequence analysis for known protein kinases, serine 177 had been predicted to be a possible site for PKA phosphorylation. For further analysis of the biological activities of the monophosphorylated variant, it was important that we identify a protein kinase which could duplicate phosphorylation at serine 177. By analogy to the PKA inhibitor protein, serine 177 of PRL may well have proven to be an additional site for PKA phosphorylation because of the presence of the Pro 11 and Arg 6 sequences to its N-terminal side (20). Analysis in the commercial PepTag assay, however, showed it to be a good substrate for PKA, but not an exceptional one. This is probably due to the presence of the Asp in the X position of the recognition sequence, RRXS. Ideally this should be a neutral amino acid.

Phosphoamino acid analysis of purified PRL phosphorylated in vitro by PKA showed only the presence of phosphoserine, consistent with only the duplication of the serine and not the threonine phosphorylation site. Prolonged trypsin digestion of PKA-phosphorylated PRL followed by HPLC on a C18 column to check for additional sites of PKA phosphorylation showed 90% of the counts to be present in flow-through peptides where they should be if phosphorylation was at the same site as in vivo (serine 177) and only 10% in one other peptide. This latter peptide was sequenced and shown to contain amino acids 131–140 of PRL (IIISQAYPEAK). Inclusion of a synthetic peptide (EKIISSQAY), representing amino acids 129–136, in an endogenous granule phosphorylation produced a mass change in the peptide from 946 to 1324, more indicative of disaccharide addition than phosphorylation. This peptide also showed no ability to compete for PKA phosphorylation in the PepTag assay. We propose therefore that this very minor site of PKA phosphorylation is an artifact of the use of very high enzyme concentrations, forcing the phosphorylation of a site normally reserved for O-linked glycosylation.

Consistent with the major phosphorylation site in vivo being at serine 177 and its duplication by PKA are the results of two-dimensional gel analysis following PKA in vitro phosphorylation of already partially phosphorylated purified PRL. This purified PRL provided by the NIDDK, is extracted from pituitary and contains non-phosphorylated, monophosphorylated, and diphosphorylated PRL. These run as charge isomers designated 2, 3, and 3’, respectively, in Fig. 8. If PKA phosphorylated a site different from those used in vivo, one would predict the production of three phosphorylated spots running as 3, 3’, and 3”. This was not seen. Only radiolabeled 3 and 3’ were observed. The highest specific activity (deduced from the size of the autoradiographic spot versus the silver-stained spot) was seen at spot 3, representing the conversion of non-phosphorylated PRL to monophosphorylated PRL. By contrast, as judged by the specificity of the diphosphorylated variant, much of the monophosphorylated material present before the reaction was unavailable for conversion to the diphosphorylated variant by PKA, thereby suggesting that the PKA site was already phosphorylated. What radioactivity is present in the diphosphorylated spot could be due to correct phosphorylation of deamidated PRL since deamidation creates a negative charge and preparations of PRL always contain some deamidated hormone. Alternatively, this could be the proposed artifactitious overphosphorylation discussed above.

It appears therefore that phosphorylation of serine 177 can
be duplicated by PKA although some care needs to be exercised so as not to overphosphorylate the molecule. To this extent, one PRL kinase is PKA-like. It remains to be determined, however, whether it resembles PKA in any other way. PKA is a cytosolic enzyme with no known mechanism to enable it to enter the secretory pathway. Cyclic AMP-dependent phosphorylation of bovine adenohypophyseal proteins has been reported previously (26) as has the association of PKA with anterior pituitary granules (27). In both cases, however, the phosphorylated granule proteins were not identified and it is not clear whether the PKA was on the cytosolic face of the granule membrane, responsible for phosphorylating proteins involved in membrane fusion during exocytosis, or inside the granule responsible for phosphorylating hormone and/or other granule constituents.

As previously mentioned, PRL can also be diphosphorylated. From the phosphoamino acid analysis we conclude that the second phosphorylation site was on a threonine. In fact, because of the relative stability of the ester bond on phosphothreonine (22, 28) and the possible inclusion of amounts of the 21-kDa protein in the excised band of these heavily loaded 10% gels, this appeared at first to be the major site. Traditional analysis by two-dimensional tryptic peptide mapping was successful in isolating this site as a discrete peptide. Mass analysis was consistent with a phosphopeptide containing two threonines. Additional analysis by two-dimensional tryptic peptide mapping was successful in isolating this site as a discrete peptide. Mass analysis was consistent with a phosphopeptide containing two threonines. Addition of this peptide containing only threonine 63 was made in an attempt to discriminate between the two threonines. Addition of this peptide to granule phosphorylation reactions did not result in phosphorylation of the peptide. This could be because phosphorylation is at threonine 58, or the peptide was too small for efficient recognition by the protein kinase. Further analysis of this minor site will require an alternate approach. It is important to note that phosphorylation of this site did not occur with PKA since PKA-phosphorylated PRL only contained phosphoserine. Thus there are apparently two PRL kinases subject to individual control. This is consistent with our earlier studies demonstrating changes in specific phosphorylated species in response to physiologic stimuli (29, 30). It should therefore be possible to reproduce both phosphorylations in vitro either separately or together as desired.

In summary we have established the primary site of PRL phosphorylation as serine 177. Phosphorylation at this site has a major effect on biological activity causing the phosphorylated PRL to become an antagonist to the non-phosphorylated hormone (8, 9). Phosphorylation at this site can be duplicated by PKA and an intragranular PKA-like enzyme may be integral to the regulation of PRL structure and function in vivo.

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