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The Rabbit Mammary Gland Prolactin Receptor Is Tyrosine-phosphorylated in Response to Prolactin *in Vivo* and *in Vitro**

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We report the first *in vivo* study demonstrating tyrosine phosphorylation of mammary gland proteins including the prolactin receptor, in response to the injection of prolactin. Immunoblotting of mammary gland membrane extracts revealed that subunits of 200, 130, 115, 100, 90, 70, and 45 kDa display increased tyrosine phosphorylation within 5 min of prolactin administration. The 100-kDa component was identified as the full-length prolactin receptor by a variety of means including immunoprecipitation and immunoblotting with monoclonal (U5, 917, 110, and 82) and polyclonal (46) antibodies to the prolactin receptor. Maximal receptor phosphorylation was seen within 1 min of hormone injection, and to obtain a strong response it was necessary to deprive rabbits of their endogenous prolactin for 36 h. Rapid tyrosine phosphorylation of the full-length receptor was verified by its demonstration in Chinese hamster ovary cells stably transfected with rabbit prolactin receptor cDNA. Both *in vivo* and *in vitro*, the phosphorylation signal was transient, being markedly reduced within 10 min of exposure to prolactin. Tyrosine-phosphorylated receptor was shown to be associated with JAK 2 by immunoblotting of receptor immunoprecipitated from transfected Chinese hamster ovary cells with polyclonal 46. A 48-kDa ATP-binding protein was also shown to be associated with the mammary gland receptor by U5 or polyclonal 46 immunoprecipitation of receptor complexes following covalent labeling with [α-^{32}P]azido-ATP.

Our demonstration of prolactin receptor tyrosine phosphorylation raises the possibility of signaling pathways regulated by receptor/SH2 protein interaction, which would facilitate prolactin specific responses. The fact that a period of hormone deprivation is needed for significant hormone triggered receptor phosphorylation indicates that the mammary gland receptor exists in a largely desensitized state *in vivo*, analogous to the related growth hormone receptor.

Despite its demonstrated role in a variety of important functions such as reproduction, osmoregulation and immune surveillance (1, 2), the mechanism of action of prolactin has remained an enigma until quite recently. Progress on this problem required the development of a new paradigm, and this stemmed from the realization that the prolactin receptor is a member of the newly recognized cytokine receptor family, which includes the receptors for growth hormone (GH),[1] erythropoietin, several interleukins, granulocyte colony-stimulating factor, and ciliary neurotrophic factor (3–6). These receptors possess common structural motifs externally, such as two disulfide loops and the WSXWS homology box, and internally, such as the proline-rich homology box 1. Based on the crystal structure of the GH $(\text{receptor})_2$ complex (7) and associated physicochemical and biological evidence (8, 9), it is believed that the initial event in signal transduction by these receptors is hormone-induced oligomerization of receptor subunits, and this brings into proximity the proline-rich box 1 of the cytoplasmic domains, which is known to be essential for signal transduction (10–12). This process is associated with the very rapid activation of a member of the JAK (Janus kinase) family of tyrosine kinases, which associate with these receptors and become activated on hormone binding (13–16). JAK activation is followed by tyrosine phosphorylation of upstream members of a variety of signaling pathways which include the cytoplasmic signal transducer and activator of transcription factor complex (16–18), and the MAP kinase pathway (19–21).

In the case of the prolactin receptor itself, some of these details are inferred, but there is biological evidence for receptor dimerization (22, 23), and rapid tyrosine phosphorylation of three proteins follows prolactin binding to the Nb2 lymphoma cell (24, 25). These three proteins comprise 97-kDa and 40-kDa components, which may be themselves kinases (25, 26), and a receptor-associated 121-kDa component, which appears to be JAK 2 (27, 28). In both reports examining prolactin-stimulated tyrosine phosphorylation in Nb2 cells, the 66-kDa mutant prolactin receptor was said to be only weakly phosphorylated (24) or not phosphorylated at all (25).

We have recently found that herbimycin A, an inhibitor of JAK 2 kinase, is able to block a substantial portion of the prolactin signal to the promoter of the milk protein gene, β-lactoglobulin, when expressed transiently with the prolactin receptor in CHO cells.[2] In order to define the role of tyrosine phosphorylation in prolactin stimulation of mammary gland function, we have examined hormone-stimulated tyrosine phosphorylation both *in vivo*, using mid-lactating rabbit mammary gland, and *in vitro*, using a stable CHO cell line expressing the full-length rabbit prolactin receptor. We find that tyrosine phosphorylation of this receptor in hormone-deprived cells is striking and very rapid, but transient, and is associated with changes in tyrosine phosphorylation of a number of cytoplasmic proteins.

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[1] The abbreviations used are: GH, growth hormone; CHO, Chinese hamster ovary; JAK, Janus kinase; mAb, monoclonal antibody; MAP, microtubule-associated protein; oPrl, ovine prolactin.

[2] N. Daniel, M. J. Waters, C. Bignon, and J. Djiane, unpublished data.

EXPERIMENTAL PROCEDURES

Materials—Ovine prolactin S15 was a gift of the National Hormone and Pituitary Program, Baltimore, MD. High titer antibody against this (19574), a gift of Dr. Ravault, Nouzilly, France, was raised in rabbits by the multiple intradermal route. Anti-prolactin receptor mAbs U5, 917, 86, and 110 have been described previously (29, 30), as has the goat polyclonal anti-rabbit prolactin receptor antibody 46 (31) and the GH receptor mAb 263 (32). Anti-trophoblastin mAb was a gift of Dr. L'Haridon, INRA, Jouy-en-Josas, France. Anti-phosphotyrosine mAb 4G10 and anti-JAK 2 were purchased from Upstate Biotechnology Inc., Lake Placid, NY. A second phosphotyrosine mAb (αPY) was produced from a hybridoma obtained from ATCC (Frackleton IG2). Peroxidase-labeled donkey anti-mouse antibody was purchased from Amersham, as were reagents for enhanced chemiluminescence. Alkaline phosphatase-conjugated rabbit anti-goat IgG was purchased from Biosys, and the substrate from Life Technologies, Inc. (Immunoselect, catalog no. 8280SA). Bromocryptine (CB 154) was a gift of Sandoz Pharmaceuticals. Protein G-Sepharose was from Pharmacia Biotech Inc. [α-^{32}P]8-azido-ATP was purchased from ICN. All other reagents were from either Sigma or Fisher and were of AR-grade.

In Vivo Studies—New Zealand White rabbits of 15 days lactation were given three subcutaneous injections of 2 mg of bromocryptine at 12-h intervals without removing the pups. At 12 h after the last injection, rabbits were given an intravenous injection of either 1 ml 150 mM NaCl or 1 mg of oPrl in saline, and after designated times (1–15 min), animals were exsanguinated and their mammary glands and livers were removed within 60 s into liquid nitrogen. Tissues were stored at −80 °C.

Cell Culture Studies—CHO K1 cells stably transfected with full-length rabbit prolactin receptor (clone E 32, selected from E3 (33) on the basis of higher binding) were maintained in Ham's F-12 with 10% fetal calf serum. Cells were grown to confluence in 10-cm dishes, and the medium was changed to GC3, a supplemented serum-free mixture of Dulbecco's modified Eagles' medium and Ham's F-12 (34) 20 h before addition of hormone. For Western blot studies, cells were scraped from dishes and resuspended at 4.5×10^7 cells/4.5 ml in GC3 medium prior to the addition of oPrl to 2 μg/ml. After designated times at 37 °C, the reaction was stopped by adding 45 ml of ice-cold phosphate-buffered saline, then cells were harvested by brief centrifugation (7 min at 200 × *g*) and resuspended in 1 ml of phosphatase inhibitor buffer minus EGTA (see below) containing 1% Nonidet P-40 and 10% glycerol, followed by rotation at 4 °C for 30 min to solubilize the membranes. The suspension was finally centrifuged at 15,000 × *g* for 10 min prior to processing the supernatant for immunoblots.

Tissue Extraction—Frozen tissues were cracked with a hammer, and 5–10-g pieces were processed as required. Solubilized membranes derived from these could be stored up to 2 weeks at −80 °C without alteration in tyrosine phosphorylation patterns. For extraction, tissue pieces were removed from −80 °C on dry ice and pulverized to a powder, which was then weighed on dry ice, and then five volumes of ice-cold phosphatase inhibitor buffer (containing 0.25 M sucrose) were added. This buffer was made freshly from stocks and consisted of 50 mM Tris, 10 mM benzamidine, 10 mM NaF, 2 mM EGTA, 5 mM sodium pyrophosphate, 20 mM sodium β-glycerophosphate, 10 μM ammonium molybdate, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μM microcystin with 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, pH 7.4, at 4 °C. Exclusion of phosphatase inhibitors led to complete loss of signal in Western blots for phosphotyrosinylated proteins. Tissue powder was homogenized with a Polytron PT20 probe in 50-ml centrifuge tubes, slowly as a frozen slurry, then at high speed once thawed, until a temperature of 6 °C was reached. The homogenate was then centrifuged at 500 × *g* for 10 min at 3 °C, and the resulting supernatant centrifuged again, at 150,000 × *g* for 40 min at 3 °C. After removal of residual fat, the pellet was resuspended in cold phosphatase inhibitor buffer diluted 1:1 with water, and Triton X-100 was added from a 10% (w/v) stock solution to a final concentration of 1%. This suspension was rotated at 20 °C for 10 min, then centrifuged again at 150,000 × *g* for 90 min at 3 °C. The clear supernatant was carefully removed from beneath the fat layer and stored in aliquots at −80 °C.

Immunoprecipitation—Solubilized tissue or cell extracts were diluted with an equal volume of phosphatase buffer to lower the Triton X-100 concentration, and 5 μg/ml anti-receptor mAb U5 or 4G10 was added (or, alternatively, 10 μl/ml polyclonal anti-receptor 46 or anti-oPrl antiserum, this being the optimum as determined by titration of signal on Western blots). Extracts were then rotated at 20 °C for 1 h, followed by centrifugation at 15,000 × *g* for 5 min at 3 °C. Fifty microliters of protein G or A-Sepharose (1:1 suspension in phosphate-buffered saline) was added to the clear supernatants, and rotation at 20 °C was continued for another 30 min. The suspension was then centrifuged at 700 × *g*, and the pellet was washed three times by resuspending in cold phosphatase buffer:wash buffer (1:3) and centrifuging. Wash buffer consisted of 20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.1% Triton X-100, pH 7.4.

Western Blots—Washed protein G or A pellets were boiled in 50 μl of Laemmli (35) sample buffer with 0.1 M dithiothreitol and the beads plus buffer were loaded onto a 1.5-mm 8% acrylamide gel (acrylamide:bisacrylamide, 29:1) and run in Laemmli buffers containing 0.5 mM sodium orthovanadate. Electrotransfer to 0.22-μm nitrocellulose was carried out at 400 mA for 4 h in 17% methanol/Laemmli running buffer at 0–4 °C. Membranes were then stained with 0.2% Ponceau S in 3% trichloroacetic acid, and after washing in water, quality of transfer verified and molecular weight markers inscribed. Following a 30-min block with 10 ml of 5% nonfat powdered milk in wash buffer (20 mM Tris, 0.15 M NaCl, 0.5 mM Na_3VO_4, and 0.1% Tween 20, pH 7.4), membranes were cut as appropriate, and placed in primary antibody in blocking solution (10 μg of antibody/10 ml for an entire membrane). After agitation for 1 h at 20 °C, membranes were agitated five times for 5 min in wash buffer, then incubated with shaking in peroxidase-conjugated anti-mouse immunoglobulins (Sigma), 1:1000 in 5% milk powder/wash buffer, for 1 h at 20 °C. After another series of five washes, location of antibody complexes was revealed by enhanced chemiluminescence as recommended by Amersham. For revelation of prolactin receptor, membranes were reblocked with milk powder as above, then incubated with polyclonal prolactin receptor antibody 46 at 1:100 in milk/wash buffer, then after the usual washes, probed with alkaline phosphatase-conjugated anti-goat IgG antibody at 1:1000 in milk/wash buffer for 30 min. Another five washes ensued, then immune complexes were localized with bromochlorophenol indophenol/nitro blue tetrazolium (Immunoselect, Life Technologies, Inc.) according to the manufacturer's instructions.

Affinity Cross-link with [α-^{32}P]Azido-ATP—Soluble extracts of mammary gland from animals injected with prolactin or vehicle were diluted with an equal volume of phosphatase inhibitor buffer containing 3 mM $MnCl_2$ and 10 mM $MgCl_2$ (without pyrophosphate, fluoride, or ammonium molybdate), and 1 ml of this was incubated on ice with 10 μCi of [α-^{32}P]azido-ATP in the presence or absence of 10 mM ATP. After 10 min, tubes were irradiated with 254 nM ultraviolet light for another 10 min, then the prolactin receptor was immunoprecipitated with U5 or 46 as described above and the washed protein A-bound immune complex was run on an 8% Laemmli gel as described for Western blots. After staining with Coomassie Blue and drying, the gel was autoradiographed with Kodak AR 5 film using intensification screens at −80 °C.

RESULTS

In Vivo Tyrosine Phosphorylation—Fig. 1*a* shows a Western blot of immunoprecipitates of tyrosine-phosphorylated proteins from extracts of rabbits injected with prolactin or vehicle alone, probed with the phosphotyrosine antibody 4G10. It is apparent that proteins of approximately 200, 130, 115, 100, 90 and 70 kDa are tyrosine-phosphorylated in response to prolactin within 2 min of injection. If a specific anti-prolactin receptor monoclonal antibody, U5, was used to immunoprecipitate the same extracts, a tyrosine-phosphorylated band at 100 kDa was seen, along with minor bands at 95 and 110 kDa (Fig. 1*a*, *right*). When the membrane was then probed with a polyclonal antibody to the prolactin receptor (S46), as can be seen in Fig. 1*b*, a major band was seen at 100 kDa and a minor band at 95 kDa. These bands are essentially absent in extracts from the animal injected with vehicle and immunoprecipitated with anti-phosphotyrosine mAb 4G10, whereas the bands are present in both extracts (± oPrl) when immunoprecipitated with the prolactin receptor mAb U5. We conclude from this that a number of proteins are rapidly tyrosine-phosphorylated in mammary gland tissue in response to prolactin, apparently including the prolactin receptor itself, which appears to exist in two forms. The tyrosine-phosphorylated 110-kDa protein associating with the receptor was seen only when monoclonal U5 was used for immunoprecipitation, and did not increase in intensity in the presence of hormone (see below).

Further Evidence That the Prolactin Receptor Is Tyrosine-

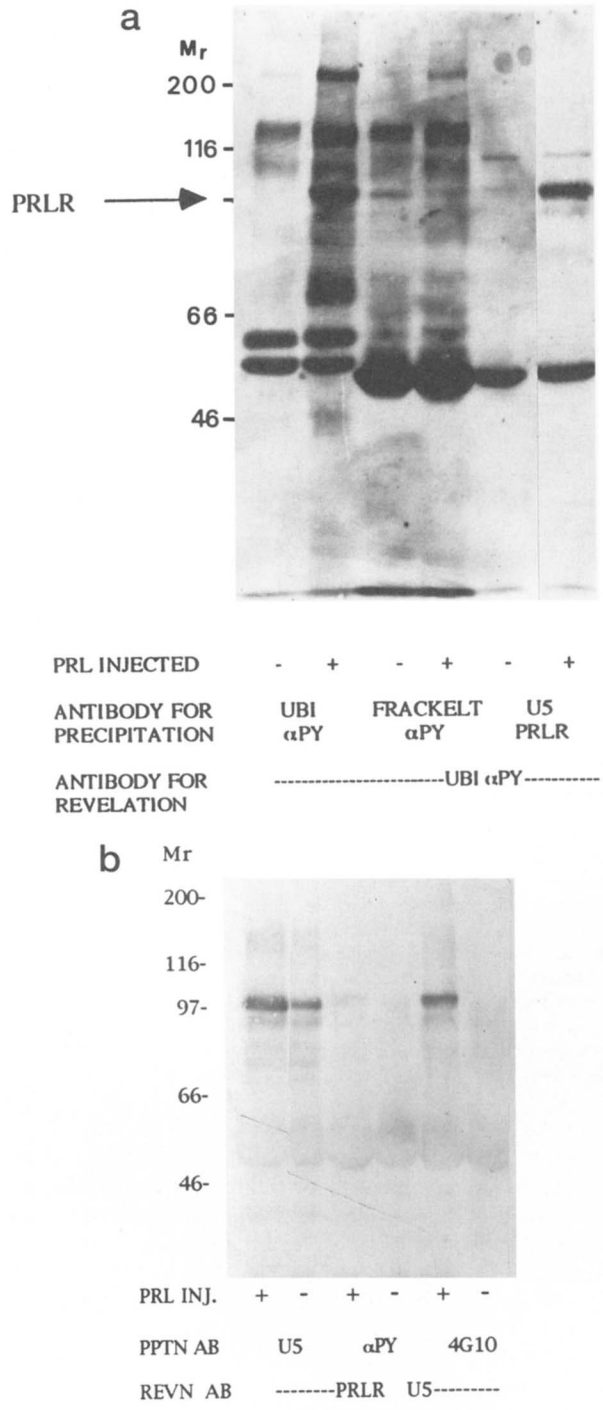

FIG. 1. **Total tyrosine phosphorylation in solubilized mammary gland membranes following prolactin injection.** 15-day lactating rabbits pretreated with bromocryptine for 36 h were injected with 1 mg of oPrl or vehicle, and after 2 min, animals were killed and solubilized membrane extracts prepared as described under "Experimental Procedures." 0.5 ml of extracts were immunoprecipitated with the phosphotyrosine mAbs UBI 4G10 (5 μg) and Frackleton αPY (35 μg), or prolactin receptor mAb U5 (5 μg). After running on an 8% reduced SDS-polyacrylamide gel electrophoresis gel, proteins were transferred to nitrocellulose and immunoblotted with 4G10 (UBI αPY) (*panel a*) or anti-prolactin receptor polyclonal 46 (*panel b*). Immune complexes were revealed as described under "Experimental Procedures." These data are representative of three experiments.

phosphorylated in Response to Prolactin—Because the full-length prolactin receptor has not previously been demonstrated in mammary gland extracts, it was prudent to establish that the 100-kDa band is in fact the receptor. Fig. 2*a* shows that when the prolactin receptor from a hormone-treated animal was immunoprecipitated with either increasing quantities of prolactin receptor mAb U5, or with three other mAbs to the rabbit prolactin receptor, and one polyclonal (S46), the same tyrosine-phosphorylated band is revealed with the 4G10. Exclusion of antibody, or use of an unrelated mAb (anti-trophoblastin) abolished the signal. Upon probing the same membrane with polyclonal receptor antibody 46, the same two bands are seen, but in this case the 95-kDa band was seen to increase in intensity relative to the phosphotyrosine blot (Fig. 2*b*). Moreover, when anti-prolactin receptor mAbs U5 and 82 were used for revelation after immunoprecipitating with U5, the same 100-kDa band was seen (Fig. 3).

In another experiment, mammary gland extracts of prolactin or saline-treated animals were immunoprecipitated with anti-ovine prolactin antiserum or with mAb U5, and the resulting immunoblot was probed for phosphotyrosine with mAb 4G10. This experiment was also designed to compare the conditions used by Argetsinger *et al.* (13) to obtain co-precipitation of JAK 2 with the GH receptor with the conditions routinely used in this study. It can be seen from Fig. 4 that while tyrosine-phosphorylated receptor is immunoprecipitated by the prolactin antibody only after prolactin administration, there is no evidence for tyrosine-phosphorylated JAK 2 associated with the receptor using either extraction and immunoprecipitation condition.

Tyrosine Phosphorylation of the Receptor in Response to Hormone Requires a Period of Prior Hormone Withdrawal and Is Transient—Fig. 5 shows that the receptor phosphorylation response of rabbits to hormone injection is markedly attenuated when the period of hormone withdrawal is only 7 h. Receptor phosphorylation at this level was seen in animals not injected with prolactin an hour after suckling (results not shown). Fig. 6 shows that the response to hormone in 20-day pregnant animals deprived of hormone for 36 h is less than that of 15-day lactating animals, and again that 8 h of deprivation is insufficient to elicit the full response to hormone. Thus a state of partial refractoriness appears to exist normally.

The time course of receptor phosphorylation in response to hormone is illustrated in Fig. 7. The response is strong after only 1 min, is maintained for 5 min, but has declined markedly by 15 min after injection. A study of the time course of phosphorylation *in vitro* using isolated mammary gland acini from a 15-day lactating animal was unsuccessful because the receptor was fully phosphorylated before addition of hormone, presumably because the enzymatic digestion procedure had preactivated the tyrosine kinase (results not shown).

Fig. 7 also shows that the livers taken from prolactin injected animals do not give a significant prolactin receptor signal in the 4G10 blots, although if the GH receptor mAb 263 was used, a diffuse band at 120–125 kDa is seen, but only in animals injected with 0.8 mg of ovine GH 5 min previously.

Time Course of Receptor Tyrosine Phosphorylation in Transfected CHO Cells—Tyrosine phosphorylation of the 100-kDa receptor subunit could not be seen in whole cell extracts of E32 cells expressing rabbit prolactin receptor (Fig. 8*a*), presumably because of the low abundance of expression (approximately 9000 receptors/cell by Scatchard analysis). These cells are prolactin-responsive (36), and there appears to be transiently increased tyrosine phosphorylation of approximately 200- and 130-kDa components after hormone exposure. After concentration of receptor by immunoprecipitation, transient tyrosine phosphorylation of the expressed receptor is clear and peaks by 1 min after exposure to a maximal dose of prolactin (Fig. 8*b*). The signal declines rapidly and is weak by 10 min after addition of hormone (Fig. 8*d*). Probing the immunoblot with poly-

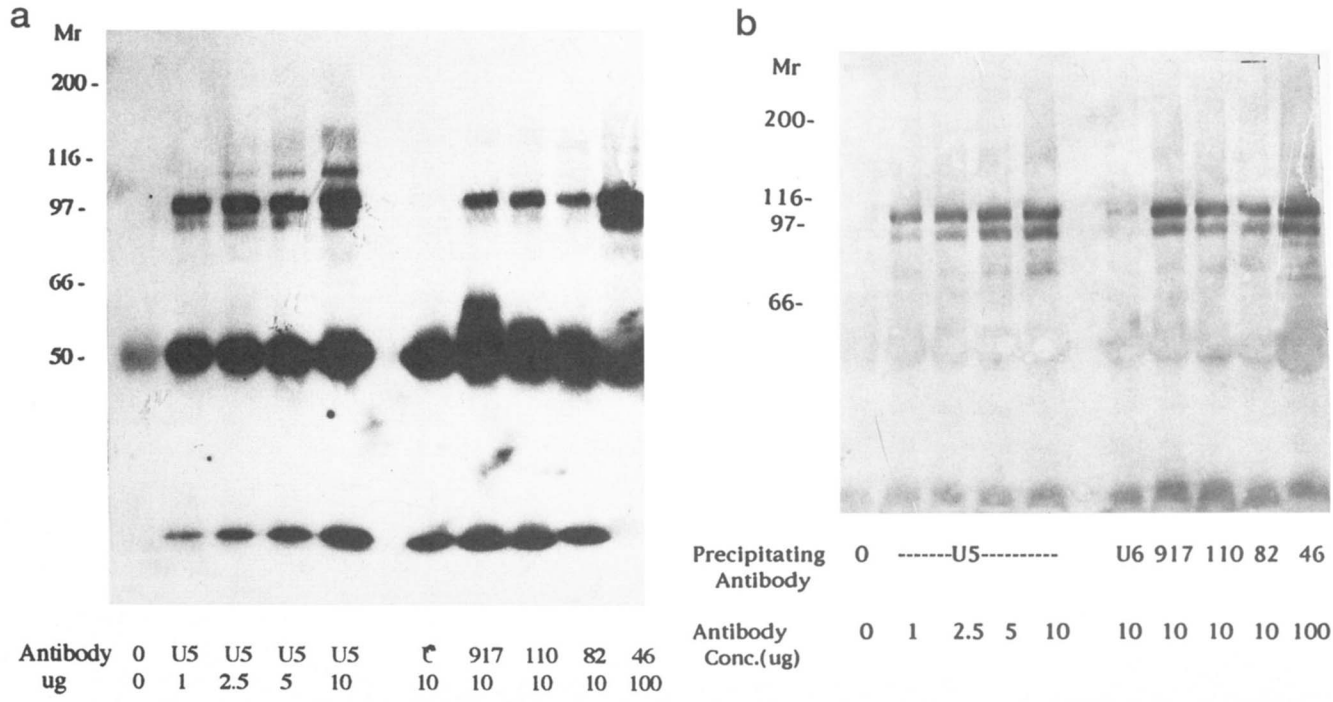

FIG. 2. **Evidence that the 100-kDa tyrosine-phosphorylated protein is the prolactin receptor.** Solubilized mammary gland extracts (0.5 ml) from a 15-day lactating rabbit injected with prolactin were immunoprecipitated with increasing quantities of receptor mAb U5, or with 5 μg of receptor mAbs 917, 110, or 82, as well as 10 μl of polyclonal 46. A control mAb to trophoblastin (*C*) showed no 100-kDa band. *Panel a*, blot revealed with anti-phosphotyrosine 4G10; *panel b*, blot revealed with anti-prolactin receptor 46. Data are representative of three experiments.

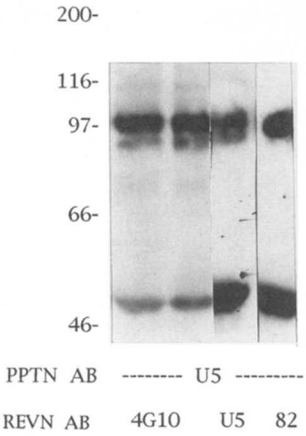

FIG. 3. **Co-localization of phosphotyrosine and prolactin receptor using enhanced chemiluminescence to reveal location of mAbs 4G10, U5, and 82 on Western blot.** Immunoprecipitation of prolactin-injected mammary gland extracts was as for Fig. 1, using 5 μg of mAb U5.

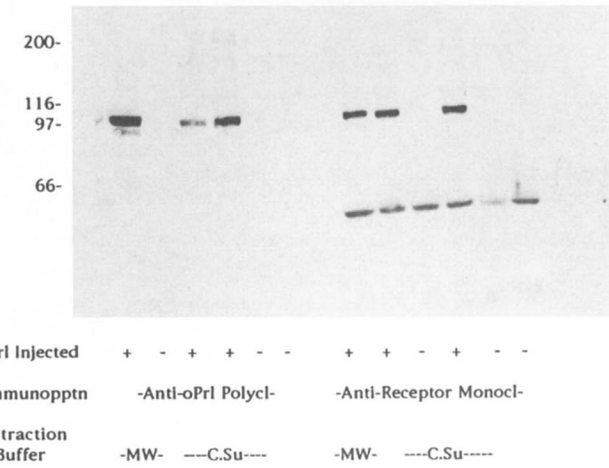

FIG. 4. **Immunoprecipitation of tyrosine-phosphorylated receptor by antiserum to ovine prolactin.** Mammary gland extracts were prepared from prolactin- or saline-injected animals either as described under "Experimental Procedures" (designated *MW*), or using the extraction buffer used by Argetsinger *et al.* (Ref. 13, designated *C-S*) but using 0.5% Triton X-100 to ensure solubilization. Complexes were immunoprecipitated either with 5 μg of U5 or with anti-oPrl at 1:1000, then processed in the usual manner and immunoblotted with 4G10.

clonal anti-receptor antibody 46 revealed that the band at 100 kDa is a receptor band, and here the band at 95 kDa is also seen to be present (Fig. 8*c*). These bands were absent in non-transfected CHO cells (data not shown).

Proteins Associating with the Prolactin Receptor—It is apparent from the preceding that a 120–130-kDa tyrosine-phosphorylated protein does not associate with the full-length prolactin receptor, either in mammary tissue or in transfected CHO cells extracted with the standard phosphatase inhibitor buffer. There is, however, evidence that a protein of this molecular mass is phosphorylated in response to hormone when whole cell extracts are immunoprecipitated with 4G10 (Fig. 1). Since a tyrosine-phosphorylated protein of 120–130 kDa has been reported to associate with the Nb2 lymphoma mutant prolactin receptor (24, 25, 28), and has been identified as JAK 2 for the prolactin, GH, and a number of other cytokine receptors (13, 14, 27, 28), we needed to establish why we could not see this association occurring.

In Fig. 9, CHO cells expressing prolactin receptor were extracted with the phosphatase inhibitor buffer without EGTA and containing 10% glycerol. Extracts were immunoprecipitated with polyclonal 46 for 3 h at 4 °C. Probing the resulting immunoblot with JAK2 antibody revealed a 125-kDa band associated with the receptor after addition of prolactin. A variety of other maneuvers, including testing six different solubilizing detergents and use of anti-oPrl for capturing activated receptor complexes, were incapable of demonstrating JAK2 association in the presence of EGTA.

Kinases possess an ATP binding site, which can be affinity-

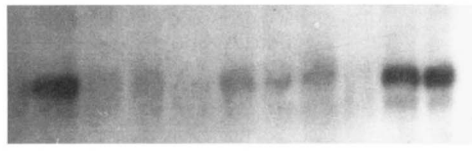

FIG. 5. **Prolonged hormone deprivation is required for maximum tyrosine phosphorylation response.** 15-day lactating animals were treated with bromocryptine for either 7 or 36 h before killing. Figure shows extracts from 10 rabbits killed 5 min after prolactin or vehicle injection and processed as in Fig. 1.

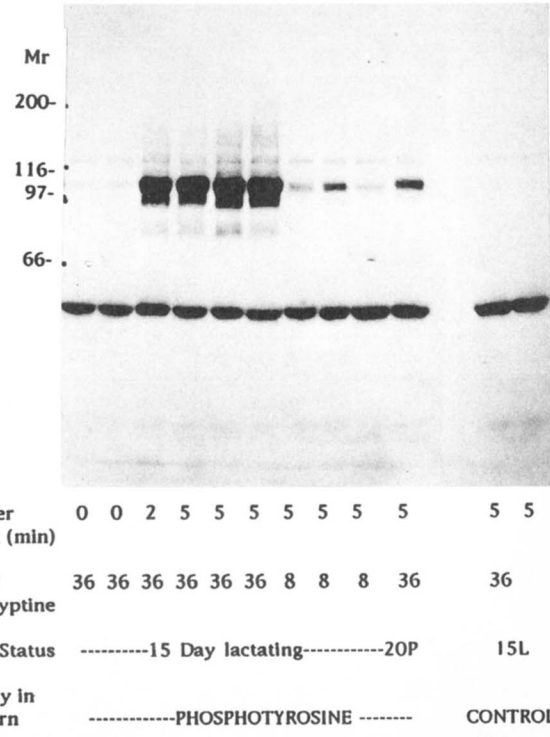

FIG. 6. ***In vivo* tyrosine phosphorylation of receptor.** Extracts from 15-day lactating or 20-day pregnant rabbits killed at different times after injection of vehicle or prolactin, with 8 or 36 h bromocryptine treatment. *Right side* of panel shows result of probing immunoblot from maximal responding animals with anti-trophoblastin mAb.

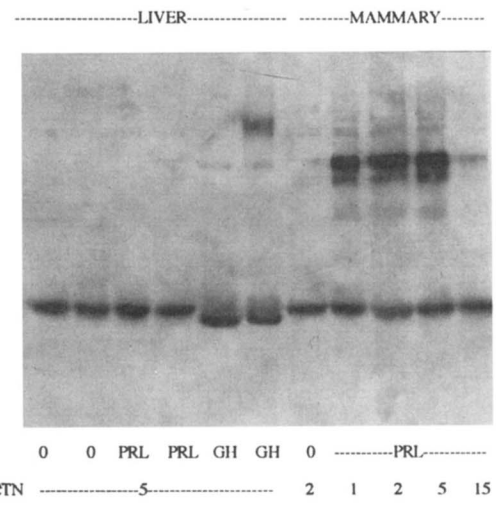

FIG. 7. **Time course of receptor phosphorylation in mammary tissue and comparison with liver.** *Right panel*, rabbits were injected with vehicle or 1 mg of NIADDK oPrl S15 and killed at the given times after injection. Mammary glands were removed and processed for immunoprecipitation by U5 followed by immunoblotting with anti-phosphotyrosine 4G10. *Left panel*, a separate group of animals were injected with vehicle, oPrl as above, or with 0.8 mg of NIADDK oGH-15. Livers were removed 5 min after injections, and after preparing solubilized microsomes as for mammary tissue, were immunoprecipitated with either U5 (oPrl-injected animals) or anti-GH receptor mAb 263 (oGH- or vehicle-injected animals). Prolactin-treated animals showed a faint prolactin receptor band in only one of the hormone-treated animals, whereas livers from ovine GH-injected animals show a diffuse band around 125 kDa corresponding to GH receptor.

labeled with azido-ATP (26), so in order to determine if other kinases were associated with the EGTA extracted receptor, we incubated mammary extracts with [^{32}P]azido-ATP, then UV cross-linked, immunoprecipitated the receptor, and examined the labeled component(s) autoradiographically after running the immunoprecipitate on a reduced Laemmli gel. Fig. 10 shows that the 100-kDa receptor is not labeled, although a 48-kDa component with an ATP binding site was found to be constitutively associated with the receptor. Association is increased by hormone treatment, however. This 48-kDa component does not appear to be a tyrosine kinase, since we have been unable to phosphorylate poly(Glu,Tyr) using protein G-bound immunoprecipitates of rabbit mammary prolactin receptor and [γ-^{32}P]ATP, even in the presence of 3 mM $MnCl_2$.[3]

DISCUSSION

This work presents what we believe to be the first analysis of *in vivo* tyrosine phosphorylation in response to administration of prolactin. It shows that the prolactin receptor is strongly tyrosine-phosphorylated within 1 min of prolactin injection, and this phosphorylation is transient, a finding confirmed with a rabbit prolactin receptor expressing CHO cell line. Moreover, there exists *in vivo* refractoriness to this phenomenon, and a prolonged period of hormone withdrawal is needed to observe the maximum extent of receptor phosphorylation. Finally, we demonstrate JAK 2 association with the prolactin receptor, as well as association with an ATP binding subunit of approximately 50 kDa.

Although the total tyrosine phosphorylation analysis was done on detergent-solubilized mammary gland membranes, it is likely that there were some residual cytoplasmic proteins, so the weaker 45-kDa band seen to be tyrosine-phosphorylated could be the MAP kinase, in agreement with the MAP kinase activation of 150–175% we have observed in the stably transfected E32 cells in response to prolactin.[3] Activation and tyrosine phosphorylation of the 42/44-kDa MAP kinase through the homologous GH receptor has been reported by others (19, 20, 37, 38), and a tyrosine-phosphorylated protein of mass around 40 kDa was reported in the Nb2 line (24) to be prolactin-sensitive. Likewise, it is plausible that the weaker band at 90 kDa is the prolactin-stimulated equivalent of the p91 component of the interferon-stimulated gene factor complex thought to be responsible for transactivation of JAK 2 responsive genes (18). The prominent band at 125–130 kDa presumably corresponds to JAK 2, as demonstrated by immunoblotting in the transfected CHO cells. This kinase was recently reported to be activated by prolactin in mouse mammary gland explants and in Nb2 cells (28). The identity of the other tyrosine-phosphorylated components at 70, 115, and 200 kDa remains to be determined.

We believe that the evidence in support of hormone-dependent tyrosine phosphorylation of the prolactin receptor is strong. First, the 100-kDa phosphorylated band co-localizes

[3] V. Mitev and M. J. Waters, unpublished results.

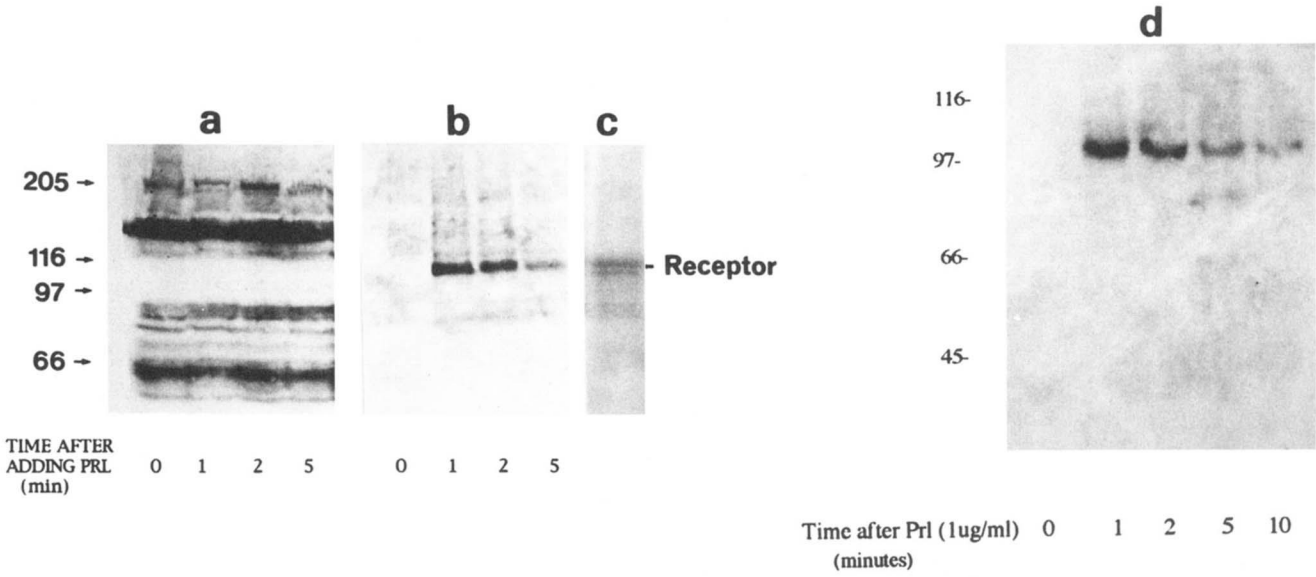

FIG. 8. **4G10 phosphotyrosine immunoblots of total cell extracts (*a*) and prolactin receptor immunoprecipitates (Ab 46) (*b* and *d*) from CHO cells stably expressing the full-length rabbit prolactin receptor.** Cells were hormone-deprived as described under "Experimental Procedures," then oPrl was added to 400 ng/ml, and after the stated times, cells were washed and harvested. The cell pellet was then either boiled directly in SDS sample buffer (*a*) or immunoprecipitated with 46 (*b*), and then both were immunoblotted with 4G10 as set out in the experimental section. In *panel c*, the membrane was also probed with Ab 46 and developed for alkaline phosphatase localization of receptor. *Panel d* is a 4G10 immunoblot of prolactin receptor polyclonal 46 immunoprecipitates from another experiment with this cell line showing the transient phosphorylation response to hormone.

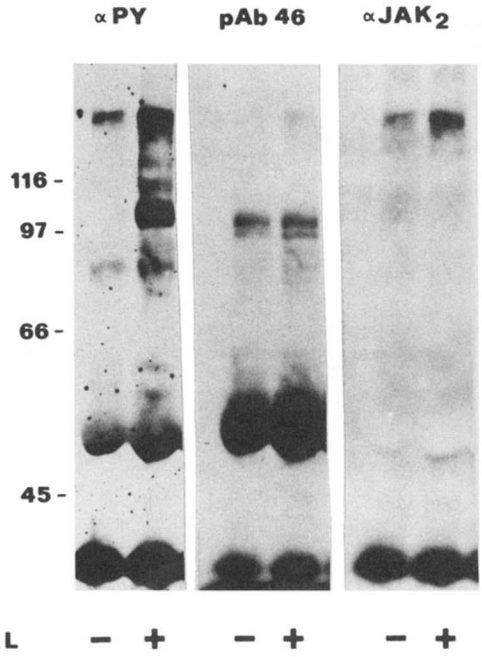

FIG. 9. **Association of JAK2 with prolactin receptor in CHO cells.** CHO cells expressing rabbit prolactin receptor were treated with prolactin or vehicle, solubilized, and immunoprecipitated with prolactin receptor antiserum S46, and the immunoprecipitates were processed for Western blotting, all as described under "Experimental Procedures." Blots were probed with 4G10, S46, or UBI JAK2 antibody and visualized by enhanced chemiluminescence.

with the receptor in Western blots using different receptor antibodies, including mAbs. Second, this phosphorylated band is seen only in CHO cells expressing the full-length receptor. Third, the 100-kDa tyrosine-phosphorylated protein is immunoprecipitable by anti-hormone antibody, but only if ovine prolactin is bound to the receptor. Finally, if the 100-kDa band were the 97-kDa cytoplasmic tyrosine kinase reported to be rapidly tyrosine-phosphorylated in response to several cytokines (26, 39), it would have been affinity-labeled by the azido-[^{32}P]ATP (24). In any case, this kinase does not immunoprecipitate with the prolactin receptor of Nb2 cells (24). We have also found that the prolactin receptor is rapidly tyrosine-phosphorylated in BAF-3 lymphoid cells stably transfected with full-length receptor cDNA (40). However, tyrosine phosphorylation of the Nb2 prolactin receptor was reported to be weak and inconsistent by Rui *et al.* (24) and was not seen by Rillema *et al.* (25, 28), yet such phosphorylation of closely homologous cytokine receptors is now firmly established (*e.g.* Refs. 13 and 41). Since Rui *et al.* (24) also used prolactin receptor antibodies to immunoprecipitate before immunoblotting for tyrosine phosphate, we need to consider why the signal we observed with the full-length rabbit receptor was so strong. Apart from the obvious differences of species and cell type, the most likely possibility is that the Nb2 receptor lacks the tyrosine residues targeted for phosphorylation because of the 198-residue deletion within its cytoplasmic domain (42). In this regard it is interesting to consider that the Nb2 line is exquisitely sensitive to prolactin, presumably because its receptor lacks this domain. This reasoning would imply that the missing domain contains a negative regulatory region analogous to that of the homologous erythropoietin receptor (41). Tyrosine phosphorylation within this domain could be the negative regulator to limit the hormone signal to a pulse, so that conversion of receptor cytoplasmic tyrosine residues to alanines, particularly tyrosines 407, 432, and 503, which lie within the Nb2 deleted 323–520 segment (43) may enhance the hormone response. Enhancement of receptor responsiveness is indeed possible, since removal of the 322–333 sequence from the rabbit receptor increases prolactin transactivation of the lactoglobulin promoter (12). Alternatively, tyrosine phosphorylation within this domain could be involved in transduction of mammary specific functions through SH2 domain containing signaling proteins such as phospholipase Cγ, Grb2, or the 85-kDa phosphatidylinositol kinase (44, 45). In support of this view, milk protein gene response to prolactin in mammary gland explants is blocked by a number of tyrosine kinase inhibitors (46). Moreover, deletion of residues carboxyl-terminal to 320 markedly

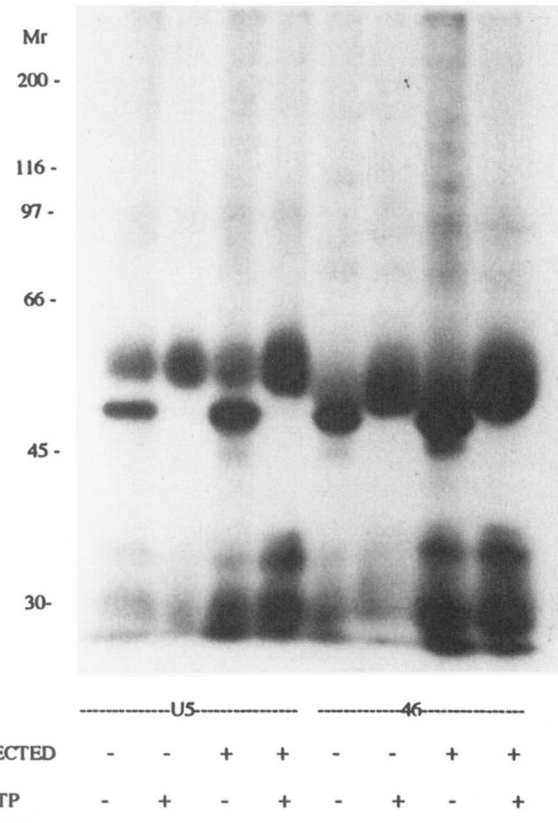

FIG. 10. **[α-^{32}P]Azido-ATP affinity labeling of receptor-associated proteins.** Lots (0.5 ml) of mammary tissue extracts from 15-day lactating animals injected with vehicle or prolactin were incubated with Mn^{2+} and Mg^{2+}, plus the photoaffinity label as described under "Experimental Procedures." Extracts were set up with or without 10 mM ATP to demonstrate specific binding. After photo cross-linking, receptors were immunoprecipitated with antibodies 46 or U5 and run on an 8% reduced SDS gel, dried, and autoradiographed as described under "Experimental Procedures."

reduces the lactoglobulin-chloramphenicol acetyltransferase response (12).

The transient nature of the receptor tyrosine phosphorylation illustrated in Figs. 7 and 8 is presumably the result of tyrosine phosphatase action, for example PTP1C (47) or PTP2 (48), which can associate with phosphorylated cytoplasmic receptor domains through SH2 interactions (49, 50). The physiological reason for a brief phosphorylation signal is unclear. Taking the argument set out above that tyrosine phosphorylation represents a "switch off" signal, phosphatase action could be seen as a means of returning the system quickly to the basal state, ready for the next hormone pulse. Alternatively, it is known in the case of the homologous interleukin 3 receptor that ligand-mediated tyrosine phosphorylation of the β subunit markedly accelerates its proteolytic cleavage (51), so that rapid dephosphorylation may be a means of reducing receptor degradation. Of course, if tyrosine phosphorylation is part of the signaling mechanism as for the epidermal growth factor receptor (52), then hormone-stimulated tyrosine phosphatase activity as for the epidermal growth factor receptor (53) could serve as a desensitization signal. One could speculate that the reason for reduced receptor phosphorylation in cells previously exposed to hormone ("desensitized") is an increased phosphatase activity, or suppression of JAK 2 activity. Indeed, the levels of these enzymes may be transcriptionally controlled by prolactin.

Finally, although we found increased tyrosine phosphorylation of a 125–130-kDa band in solubilized mammary membranes from prolactin-treated animals, we were only able to show association of JAK 2 with the receptor when EGTA was omitted and glycerol was included in the extraction buffer. We have also observed a 48-kDa ATP binding subunit, which associates with the prolactin receptor, even in the absence of hormone. This protein does not appear to be a tyrosine kinase, based on our inability to obtain phosphorylation of poly(Glu,Tyr) *in vitro* with immunoprecipitated hormone-receptor complexes, and may represent a novel serine/threonine kinase. We are currently pursuing the identification of this interesting protein.

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