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Dissociation of Janus Kinase 2 and Signal Transducer and Activator of Transcription 5 Activation after Treatment of Nb2 Cells with a Molecular Mimic of Phosphorylated Prolactin*

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ABSTRACT

We have previously demonstrated that phosphorylated PRL acts as an antagonist to the Nb2 proliferative activities of unmodified PRL. A molecular mimic of phosphorylated PRL, which substitutes an aspartate residue for the normally phosphorylated serine (serine 179), has the same properties. Because it takes less than one fourth the amount of phosphorylated hormone, or the aspartate mutant, to block the proliferative activity of unmodified hormone, we have investigated whether the high potency of the aspartate mutant is achieved by the production of an alternate and interfering intracellular signal cascade. Nb2 cells were exposed to 5 or 500 ng/ml human NIDDK PRL, wild-type recombinant PRL (unmodified PRL), or aspartate mutant PRL (pseudophosphorylated PRL) for 1, 5, or 10 min at 37 C. At 5 ng/ml and 10 min, wild-type recombinant PRL showed greater activation of Janus kinase 2 (JAK 2) than the NIDDK preparation. This is consistent with a previous report of higher proliferative activity for the wild-type hormone and is primarily a reflection of the presence of some phosphorylated hormone in the NIDDK preparation. At 500 ng/ml and 10 min, saturation eliminated any differences between responses to the two preparations. JAK 2 activation was not seen in response to the aspartate mutant at either concentration. Signal transducer and activator of transcription 5 (STAT 5) activation was, however, just as robust for the aspartate-treated cells as for the other two groups. Time course experiments eliminated the possibility that STAT 5 phosphorylation in response to the aspartate mutant was the result of JAK 2 activation at earlier time points. Experiments in the present study also interestingly showed preassociation of JAK 2 and STAT 5 in the absence of PRL and the absence of detectable phosphorylation of either JAK 2 or STAT 5. Like JAK 2, receptor phosphorylation was absent with the aspartate mutant. A comparison between STAT 5a and STAT 5b activation showed a marked reduction in STAT 5b phosphorylation in response to the aspartate mutant, with concomitant reduction in STAT 5a-STAT 5b heterodimers. STAT 5a activation, however, was indistinguishable between the wild-type and aspartate mutant. We conclude that the nonproliferative aspartate mutant signals and activates STAT 5 without, or with minimal, use of JAK 2 or receptor phosphorylation. The wild-type proliferative PRL, on the other hand, uses receptor phosphorylation and JAK 2 activation. (*Endocrinology* **140:** 5087–5094, 1999)

PRL, A HORMONE originally named for its role in milk production, is a member of a large, hormone/cytokine family (1–3). Other members of the family include GH, erythropoietin, granulocyte-macrophage colony-stimulating factor, and a number of interleukins (3). Members of the family have structural similarities, as do their cognate receptors (1–4). The receptors/receptor subunits are single transmembrane domain proteins without intrinsic tyrosine kinase activity (2, 4). Ligands in this family bind to two or more receptors/receptor subunits, which themselves associate with one or more signal-transducing proteins (1–4).

For PRL, homodimerization of the PRL receptor (PRL-R) is thought to result from PRL binding and to cause activation of a constitutively-associated tyrosine kinase, Janus kinase 2 (JAK 2) (5–8). This activation of JAK 2 is followed by tyrosine phosphorylation of the PRL-R, a step thought to be involved in efficient recruitment of a transcription factor, signal transducer and activator of transcription 5 (STAT 5), to the transduction complex (9). STAT 5 is then thought to be phosphorylated by JAK 2, to dimerize and then enter the nucleus (10). STAT 5 belongs to a family of transcription factors with seven members to date, STATs 1–4, 5a, 5b, and 6 (10–14). In addition, STATs may also have splice variants, contributing to the potential diversity of their roles (15). Most STATs have at least semispecific roles in the immune system. STATs 1 and 2, for example, are critical for viral resistance, STAT 6 mediates the effect of interleukin 4, and STAT 4 is critical for interleukin 12 signaling. STAT 5, on the other hand, is activated by a wide variety of cytokines in addition to PRL. These include GH, erythropoietin, thrombopoietin, interleukins 2 and 3, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor (13–15). These cytokines can all activate the DNA-binding ability of STAT 5 and/or transactivate a β-casein luciferase reporter gene *in vitro* but are unlikely to be involved in modulation of milk protein gene expres-

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sion *in vivo* (16). Thus, there must be subtleties to STAT 5 activation, which may include differential activation of 5a and 5b, activation of splice variants of each, and coactivation of other STATs and other transcription factors.

PRL is produced in a number of posttranslationally modified forms (17). Phosphorylated PRL, which is quantitatively the most important posttranslationally modified product of the pituitary (18), has been shown to have antagonistic effects to unmodified hormone (19–21). Thus, unmodified PRL stimulates cell proliferation, whereas phosphorylated PRL blocks this response (20, 21). To more closely study the effects of phosphorylated PRL, we have produced a recombinant molecular mimic of phosphorylated PRL, which duplicates the antagonistic effects of naturally phosphorylated PRL on PRL-driven cell proliferation (22). The molecular mimic, produced by the substitution of an aspartate residue for the normally phosphorylated serine, has several advantages over the use of phosphorylated material. The most important advantage is removal of the possibility of conversion of phosphorylated PRL to unmodified PRL during the course of an experiment.

As an antagonist, both phosphorylated hormone and the molecular mimic are extremely potent (21, 22). This is shown by the fact that it takes less than one quarter the amount of each to completely block the proliferative activity of unmodified PRL (21, 22). This efficacy led us to consider the possibility that antagonism was not simply attributable to blockade of the unmodified PRL signal transduction pathways but may involve the generation of alternate intracellular signals. In addition, we hypothesized that phosphorylated PRL had a set of biological activities, beyond antagonism of PRL-stimulated cell proliferation, which require appropriate signal transduction pathways.

Materials and Methods

Cells

Nb2 cells were originally obtained from Henry Friesen (now at Medical Research Council, Ottawa, Canada). These are T lymphoma cells which respond to lactogens by proliferating (23). Proliferation of Nb2 cells is now the most widely used measure of PRL bioactivity. Cells were routinely cultured in Fischer's medium containing 10% FBS, 10% HS, 0.1 mM $NaHCO_3$, 0.1 mM β-mercaptoethanol, and penicillin (20 U/ml)/streptomycin (20 μg/ml) (complete medium).

Recombinant protein expression

Recombinant wild-type human PRL (hPRL) and the molecular mimic of phosphorylated PRL (hPRL aspartate mutant) were prepared, as previously described, by expression in *Escherichia coli* (22). The aspartate mutant substitutes an aspartate for serine 179. To assure comparability in the protein preparations, wild-type PRL and the aspartate mutant were expressed at the same time and isolated and refolded in parallel (22). The proteins were then tested for biological activity (proliferative or antiproliferative, as appropriate) in an Nb2 bioassay and compared with an NIDDK (B3AFP3855A) preparation of hPRL (22). The wild-type recombinant hPRL routinely had a higher biological activity than the NIDDK PRL (22). Proteins were quantified by gel densitometry, using NIDDK PRL to produce a standard curve. These proteins have previously been tested for endotoxin contamination, and none was detectable (22). Antagonist activity is also reversible by the addition of excess wild-type hormone.

PRL treatment of the cells

Cells were placed in lactogen-free medium (complete medium without FBS) 16–18 h before exposure to the purified PRL preparations. At the time of the experiment, cells were pelleted and aliquoted in lactogen-free medium at 10^8 cells/ml. One ml was placed into 1.5-ml centrifuge tubes and allowed to equilibrate/recover for at least 30 min at 37 C before PRL exposure. Cells were then exposed to prewarmed (37 C) PRL solutions in the same medium by the addition of 100 μl of 11× concentrated material. Mixing was assured by gentle inversion of the tube. PRL concentrations and times of exposure are indicated in the figures. At the end of the appropriate exposure, cells were washed twice with ice-cold stop buffer [20 mM Tris (pH 7.4); 150 mM NaCl with 10 mM each of sodium pyrophosphate, sodium fluoride, and sodium vanadate; 10 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM PMSF; 0.02% sodium azide; and 1 mM EDTA] and then lysed by resuspension in 1 ml of 1% Triton X-100 in the same buffer. After a 1-h rotation of the tubes at 4 C, lysates were subjected to centrifugation at 13,000 × *g* for 10 min, and the supernatants were saved for immunoprecipitation.

Immunoprecipitation

Total lysates were used for immunoprecipitation. Antibodies were as follows: polyclonal anti-JAK 2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); polyclonal anti-STAT 5b (Santa Cruz Biotechnology, Inc., recognizes both STAT 5a and STAT 5b and hence is described throughout as antipan STAT 5); polyclonal anti-STAT 5a (Santa Cruz Biotechnology, Inc., specific for STAT 5a); polyclonal anti-STAT 5b (Zymed Laboratories, Inc., San Francisco, CA, specific for STAT 5b). Four micrograms of purified antibody was added to 1 ml of lysate and incubated for 2 h at 4 C. Anti-PRL-R serum (no. 120) was kindly provided by Patricia Ingleton (Sheffield University, United Kingdom), and 30 μl of whole serum was used per milliliter of lysate. This antibody was raised against rat liver receptor extracellular domain (24). Antibodies were precipitated by incubation for 1 h further at 4 C after addition of 8 μl washed protein G Sepharose slurry (Amersham Pharmacia Biotech, Piscataway, NJ). Preliminary experiments determined that preclearing of the lysates by precipitation with nonspecific antibodies was unnecessary. After precipitation with protein G, the pellets were washed three times in stop buffer and then incubated in reducing SDS sample buffer at 95 C for 10 min before loading on a 7.5% polyacrylamide gel. Usually, minigels were used with electrophoresis at 40 mA constant current for 1 h. Results from the use of 11-cm gels, run at 60 mA for 3 h, are shown in Figs. 4 and 5.

Western blot

After protein transfer onto nitrocellulose membranes in 48 mM Trizma, 39 mM glycine, 0.1% SDS, and 10% methanol (pH 8.3), membranes were blocked with 10% nonfat milk in wash buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% BSA, 0.1% Tween 20). Blocked membranes were probed with antiphosphotyrosine (Upstate Biotechnology, Inc. Lake Placid, NY), diluted 1:2000 in wash buffer for 2 h at room temperature. After washing 3 times for 15 min, the blot was reprobed with goat antimouse conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) at 1:2000 for 1 h at room temperature. After 5 washes, horseradish peroxidase-positive bands were detected with ECL reagent (Amersham Pharmacia Biotech) followed by autoradiography, with 1- to 10-sec exposure times. Blots were then stripped for 1 h at 70 C in 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris, pH 6.8. After blocking and washing, membranes were reprobed with anti-JAK 2 and/or one of the anti-STAT 5 diluted 1:1000 in wash buffer. This time, antibodies were localized by using goat antirabbit conjugated to horseradish peroxidase (Sigma Chemical Co.) at 1:2000 and the ECL reagent. Every blot included a positive control for antiphosphotyrosine antibodies (Upstate Biotechnology, Inc.), which also served as a control for thorough stripping of the antiphosphotyrosine. Additional controls included tests of the second antibodies alone for each of the immunoprecipitating antibodies. Proteins were identified by overlaying the autoradiograms derived from the sequential blots.

Results

Using NIDDK hPRL B3, preliminary experiments determined a dose response relationship between PRL concentration and the degree of JAK 2 and STAT 5 phosphorylation. Under the conditions used, a faint phosphorylated JAK 2 band was detectable at 0.5 ng/ml (~0.02 nM), and a maximum response was achieved at 500 ng/ml (~20 nM, Fig. 1A). Concentrations up to 3.5 μg/ml were tested (higher concentrations not shown). Reprobing with a mixture of anti-JAK 2 and antipan STAT 5 showed equivalent loading of the lanes (B). Similar dose response relationships were seen between PRL and STAT 5 activation (C). Again, equivalent loading is demonstrated (D). To compare the signaling response between the wild-type recombinant PRL and NIDDK PRL, a nonmaximum dose of 5 ng/ml was chosen. Fig. 2A shows phosphorylation of JAK 2 in response to either NIDDK PRL or the recombinant wild-type PRL. At a dose of 5 ng/ml, the wild-type PRL results in a greater degree of JAK 2 phosphorylation. This is consistent with a higher biological activity in the Nb2 bioassay previously described (22) (see later for further discussion). At this concentration of PRL, no differences were detected between the two preparations, in terms of their ability to cause the phosphorylation of STAT 5 (Fig. 2C). Eqivalent loading of lanes is demonstrated in B and D, which are stripped and reprobed versions of A and C, respectively. B shows immunoprecipitation with anti-JAK 2 followed by blotting with a mixture of anti-JAK 2 and antipan STAT 5 antibodies. D shows immunoprecipitation with antipan STAT 5 antibody followed by blotting with the same mixture. E is equivalent to B, except for the use of only one antibody for Western blotting. F is a stripped and reprobed version of E, showing even loading. G shows a composite of overexposed second-antibody controls, which demonstrate the specificity of staining.

Of interest in the first and second figures is also evidence of JAK 2-STAT 5 association in the absence of PRL stimulation or phosphorylation of either JAK 2 or STAT 5. This can be seen in the O PRL lane on Fig. 1 by comparing A (showing no phosphorylation) and B (showing immunoprecipitation of STAT 5 with anti-JAK 2) and in the C lanes of Fig. 2. It is more obvious in Fig. 2B than 2D, probably because of the relative quantity of JAK 2 *vs.* STAT 5. When anti-JAK 2 is used to precipitate, a substantial band of STAT 5 is evident. When antipan STAT 5 is used to precipitate, a smaller band of JAK 2 is evident. This preassociation result is unlikely to be attributable to cross-reactivity of anti-JAK 2 and antipan STAT 5 antibodies, because both were produced against synthetic peptides corresponding to specific regions of the appropriate molecules. Additional controls demonstrated that it is not an artifact caused by coblotting with anti-JAK 2 and antipan STAT 5, because blotting with only one antibody gave the same result (E and F). G shows that this is not an artifact caused by second-antibody staining. Similar preassociation of JAK 2 and STAT 5 has also been observed in our studies in both the mouse and bovine mammary cell lines, 31EG4 and MAC T (data not shown).

At the higher dose of 500 ng/ml, JAK 2 phosphorylation reached a plateau, and it was no longer possible to discern the difference between wild-type recombinant and NIDDK PRL (Fig. 3). The higher concentration was used, however, to stringently test for signaling of the aspartate mutant. A quantity of 500 ng/ml aspartate mutant produced minimal JAK 2 phosphorylation (A) but almost equivalent phosphorylation of STAT 5 (C). Consistent with minimal activation of JAK 2 in response to the aspartate mutant is the absence of phosphorylated JAK 2 in C after immunoprecipitation with antipan STAT 5.

To eliminate the possibility that JAK 2 phosphorylation occurred earlier in the aspartate-treated cells, a time course of JAK 2 and STAT 5 phosphorylation was conducted (Fig. 4). Only minimal JAK 2 phosphorylation occurred in response to the aspartate mutant at 1 and 5 min, as well as at the 10-min time point previously used. STAT 5 phosphorylation, on the other hand, was robust at all time points. Preassociation of JAK 2 and STAT 5 in the absence of PRL can

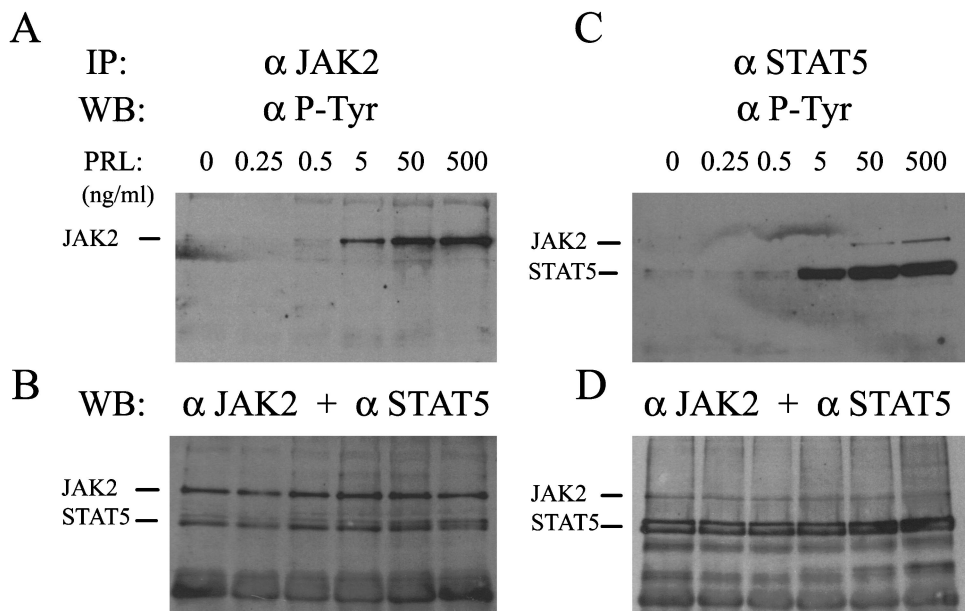

FIG. 1. JAK 2-STAT 5 dose response to NIDDK PRL. Nb2 cells were exposed to NIDDK PRL for 10 min at the concentrations indicated. Cell lysates were subjected to immunoprecipitation (IP) with either anti-JAK2 (α JAK 2) or antipan STAT 5 (α STAT 5) and then Western blotted (WB) with antiphosphotyrosine (αP-Tyr) (A and C). Blots were then stripped and reprobed with a mixture of anti-JAK 2 and anti-STAT 5 (B and D).

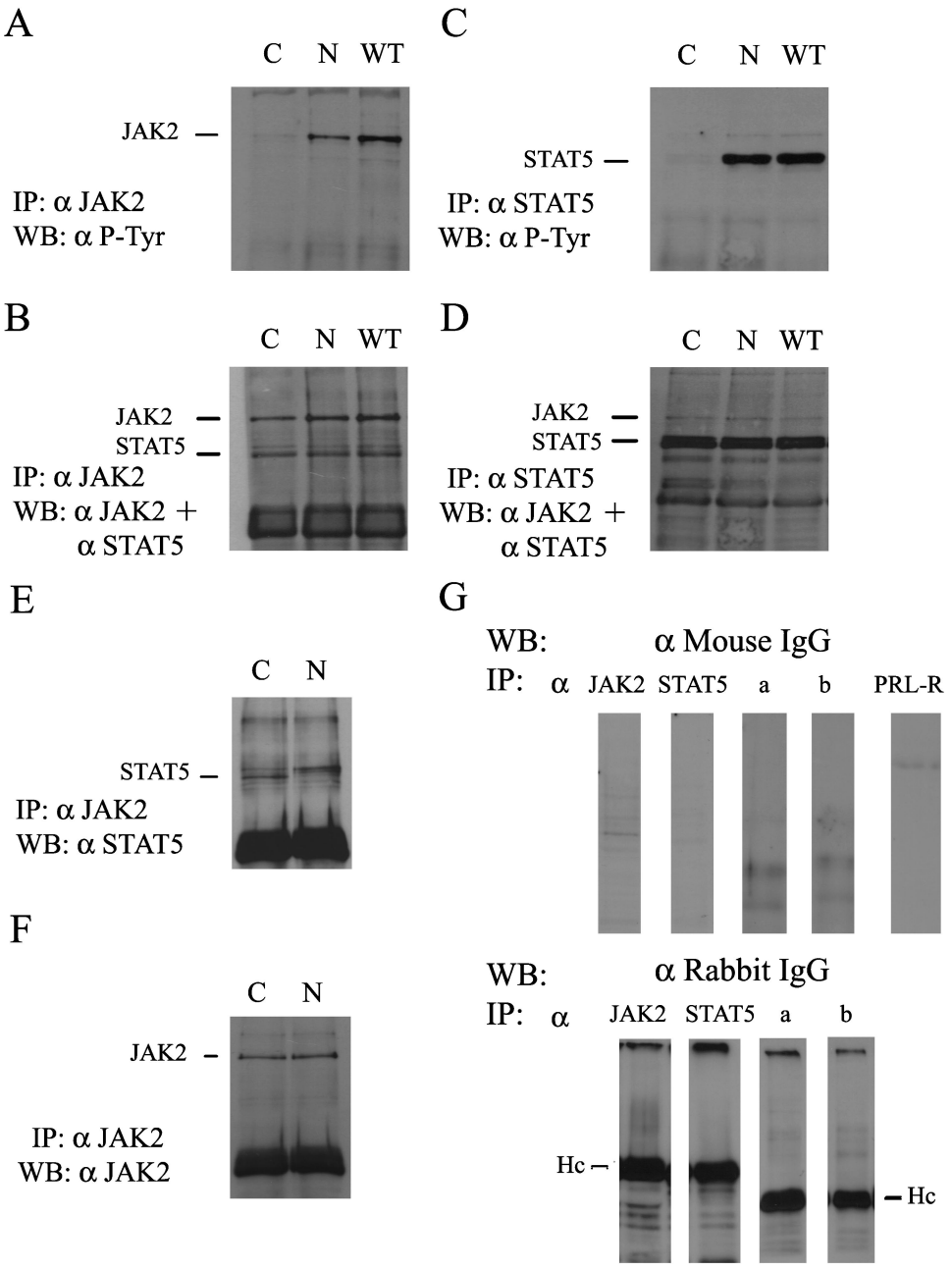

FIG. 2. Phosphorylation of JAK 2 and STAT 5 in response to stimulation with 5 ng/ml (panels A–D) or 500 ng/ml (E–G) NIDDK PRL or wild-type recombinant PRL for 10 min. Cells were stimulated with vehicle as control (letter C, marking *left* lanes), NIDDK PRL (N, marking *middle* lanes), or wild-type recombinant protein (WT, marking *right* lanes) for 10 min. Cell lysates were subjected to immunoprecipitation with anti-JAK 2 (A and B) or antipan STAT 5 (C an D) and then Western blotting with antiphosphotyrosine (A and C). After stripping, the blots shown in panels A and C were reprobed with a mixture of anti-JAK 2 and antipan STAT 5. For E, lysates were precipitated with anti-JAK 2 and membranes probed with antipan STAT 5 only. This blot was stripped and probed with anti-JAK 2 (F). E and F control for any possible artifact produced by coprobing for JAK 2 and STAT 5, as was done to produce B and D. A and B were from 1 experiment, C and D from another, and E and F from yet another. The JAK 2 and STAT 5 bands, identified by Western blotting, therefore ran at slightly different distances from one another. G shows a composite of multiple experiments to test the specificity of the secondary antibodies used in Western blotting throughout the manuscript. Cell lysates were subjected to immunoprecipitation with the primary antibodies indicated directly above each lane. STAT 5, Antipan STAT 5; a, anti-STAT 5a; b, anti-STAT 5b. Western blots used antimouse IgG (α mouse IgG) for the *upper panels* and antirabbit IgG (α rabbit IgG) for the *lower panels*.

again be seen in B. In these larger gels, STAT 5 resolves into several bands.

When titrated against one another, the aspartate mutant competes with wild-type hormone for JAK 2 activation (Fig. 5).

Because receptor phosphorylation is considered an integral part of PRL signal transduction, we also examined receptor phosphorylation in response to NIDDK PRL B3, wild-type recombinant PRL and the aspartate mutant. Figure 6 shows phosphorylation of the receptor in response to NIDDK PRL and wild-type recombinant and very little, if any, receptor phosphorylation in response to the aspartate mutant.

Because there are two main forms of STAT 5 associated with PRL signal transduction, we investigated their relative tyrosine phosphorylation in response to wild-type and aspartate mutant. Figure 7A shows essentially equivalent phosphorylation of STAT 5a in response to either wild-type or aspartate mutant, whereas phosphorylation of STAT 5b in response to the aspartate mutant was much reduced. Because of this, there was also reduced STAT 5a-STAT 5b heterodimerization, as shown on the *right side* of B. Consistent immunoprecipitation of STAT 5b is illustrated in C (*right side*).

Discussion

Others have determined the involvement of JAK 2 and STAT 5 in PRL signal transduction (5–12). Our results, using NIDDK PRL, as expected, confirm JAK 2 and STAT 5 phos-

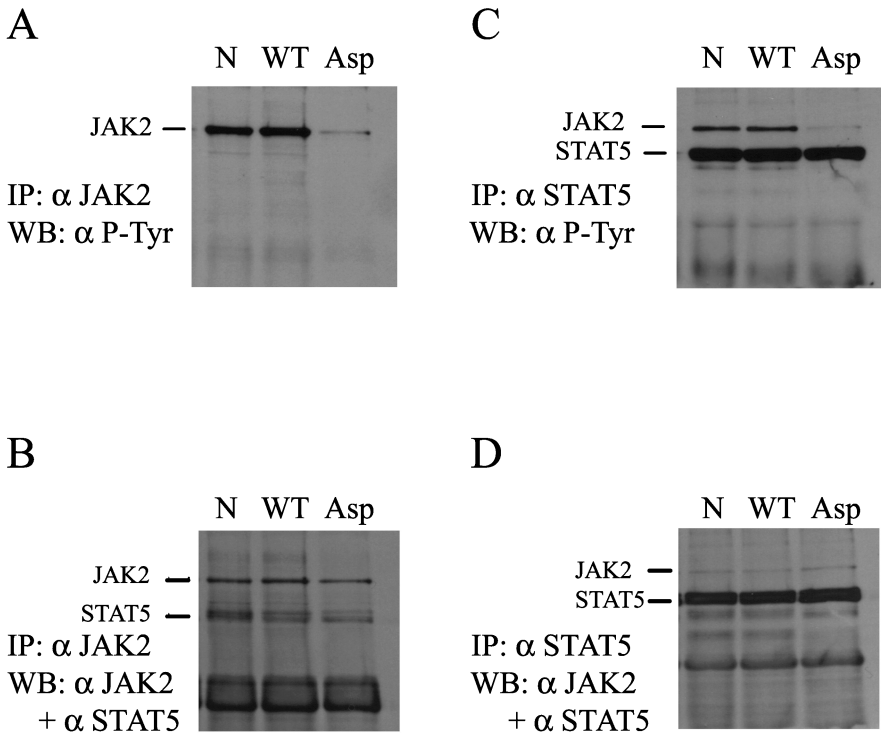

FIG. 3. Phosphorylation of JAK 2 and STAT 5 in response to stimulation with 500 ng/ml NIDDK PRL (N), wild-type recombinant PRL (WT), or the aspartate mutant PRL (Asp) for 10 min. B and D are reprobed versions of A and C, respectively. All other notations are the same as previous figures.

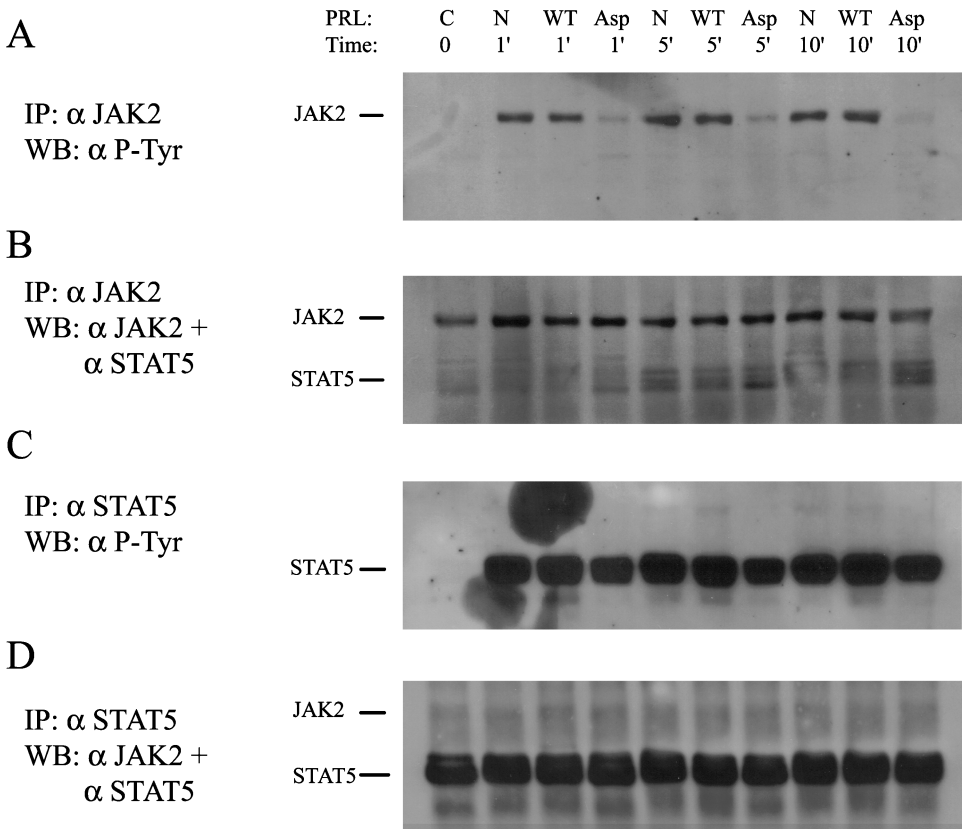

FIG. 4. Time course of JAK 2 and STAT 5 phosphorylation in response to 500 ng/ml NIDDK PRL, wild-type recombinant PRL, or aspartate mutant PRL. B and D are once again reprobed versions of A and C, respectively.

phorylation after stimulation of Nb2 cells with PRL. NIDDK hPRL is extracted from pituitaries, post mortem. It contains glycosylated (25) and phosphorylated PRL (22), in addition to unmodified hormone. The presence of both glycosylated and phosphorylated forms reduces the Nb2 proliferative activity of the overall preparation because glycosylated PRL

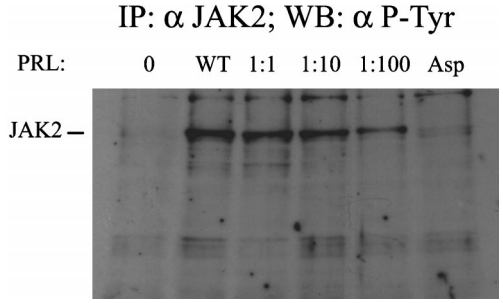

FIG. 5. Phosphorylation of JAK 2 in response to wild-type and aspartate mutant titrations. Cells were exposed to no PRL (0), wild-type PRL only (WT) at 500 ng/ml, equal concentrations of wild-type and aspartate mutant (1:1), excess aspartate mutant at 10-fold (1:10), or 100-fold (1:100) the wild-type, or aspartate mutant alone (Asp) at 500 ng/ml for 10 min before lysis and immunoprecipitation with α JAK 2, followed by Western blotting with antiphosphotyrosine (α P-Tyr).

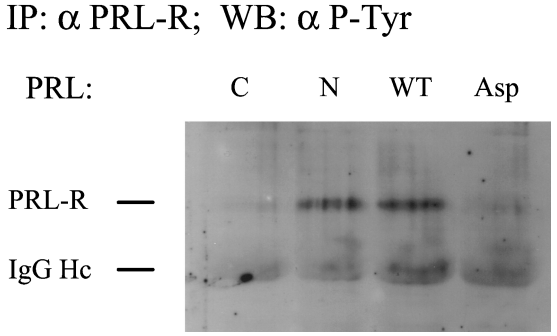

FIG. 6. Phosphorylation of the PRL-R in response to the different PRLs. Cells were incubated in 500 ng/ml of the different PRLs for 5 min. Cell lysates were subjected to immunoprecipitation with anti-PRL-R (αPRL-R) and then Western blotting with antiphosphotyrosine. IgG Hc marks the position of the Ig heavy chain.

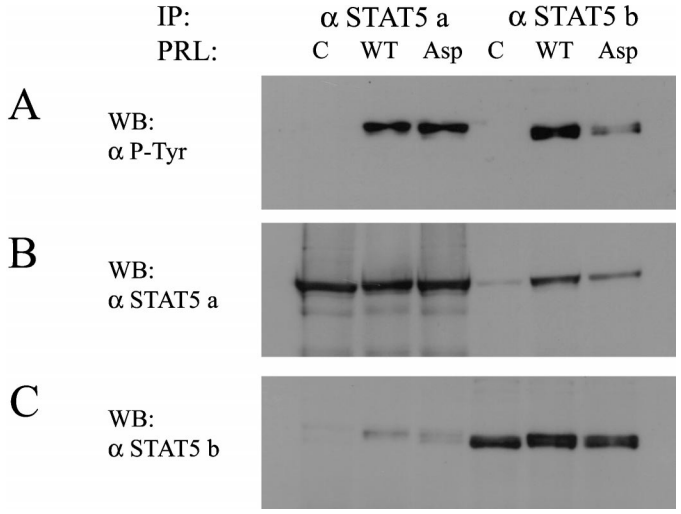

FIG. 7. Phosphorylation of STAT 5 isoforms in response to the different PRLs. Cells were exposed to 500 ng/ml of each PRL under identical conditions to those used to produce Figs. 2 and 3 (a 10-min incubation). Half of the cell lysate was then used for immunoprecipitation with α STAT 5a and half for immunoprecipitation with α STAT 5b. Two identical gels were then run and Western blotted with antiphosphotyrosine (only one shown in A). These were then stripped and reprobed with anti-STAT 5a (B) or anti-STAT 5b (C).

has a reduced Nb2 proliferative activity (26) and phosphorylated PRL acts as an antagonist to the proliferative activity of the unmodified hormone (21). When equivalent total amounts of recombinant wild-type PRL and NIDDK PRL were therefore used in the signaling studies, it was not surprising to find a greater degree of JAK 2 phosphorylation in response to recombinant wild-type PRL, as shown in Fig. 2A. This is in accord with previous reports from this lab of greater proliferative activity of the recombinant wild-type *vs.* NIDDK PRL in the Nb2 bioassay (22) and supports the role of JAK 2 in PRL signaling leading to cell proliferation. Likewise, the essential absence of JAK 2 phosphorylation in response to the aspartate mutant would also support this concept: a molecule, mimicking a proliferation antagonist (phosphorylated PRL), would not be expected to phosphorylate JAK 2. By competition experiments, it can also be shown that the aspartate mutant blocks the ability of wild-type PRL to cause phosphorylation of JAK 2, and we know that the aspartate mutant blocks cell proliferation by wild-type hormone (21).

Surprisingly, however, the aspartate mutant results in indistinguishable levels of STAT 5 phosphorylation when compared with NIDDK or recombinant wild-type hormone. To be sure that this result was not simply the consequence of an altered time course of JAK 2 activation, a time course experiment ranging from 1–10 min was conducted. As before, there was essentially no activation of JAK 2. It seems therefore that STAT 5 can be activated in Nb2 cells without, or with very minimal, use of JAK 2. This result demonstrates that the aspartate mutant not only blocks signaling from wild-type hormone but also signals via an alternate pathway.

Because STAT 5 was equally activated by the aspartate mutant and the aspartate mutant is antiproliferative, this result also suggests that STAT 5 activation can result from both proliferative and nonproliferative signaling from the PRL-R. What receptor-associated kinase is responsible for STAT 5 activation in response to the aspartate mutant is currently the subject of investigation. Other investigators have reported an association between the PRL-R and other tyrosine kinases (27–29) and activation of some in response to NIDDK PRL (27–29). Because NIDDK PRL contains both unmodified PRL (equivalent to wild-type) and phosphorylated PRL (equivalent to aspartate mutant), one would expect use of both proliferative and nonproliferative signaling pathways. STAT 5 phosphorylation by one of these previously described kinases, in response to the aspartate mutant, therefore is a reasonable possibility.

Receptor phosphorylation is considered important in efficient recruitment of STAT 5 to the transduction complex (9, 30). When examining receptor phosphorylation, we found no detectable phosphorylation of the receptor in response to the aspartate mutant. Thus, it seems that, with the aspartate mutant, we have efficient STAT 5 phosphorylation in the absence of receptor phosphorylation. Certainly, STAT 5 can be phosphorylated in the absence of receptor phosphorylation (31–33), but the current results suggest the existence of an alternate mechanism for efficient recruitment of STAT 5 to the transduction complex. This is consistent with previous reports, in PRL signaling and homologous systems, showing the presence of two distinct mechanisms of STAT 5 activa-

tion: one dependent on receptor tyrosine phosphorylation and the other not (31–34).

Another unexpected finding in the current study was evidence of JAK 2 and STAT 5 association before any addition of PRL or phosphorylation of either JAK 2 or STAT 5. This implies an interaction between JAK 2 and STAT 5 without the involvement of SH2 domain-phosphotyrosine interactions. A search of the literature found no other description of an experiment in which both JAK 2 and STAT 5 had been localized by Western blot after immunoprecipitation for either JAK 2 or STAT 5. This is presumably the reason why such preassociation has not been previously reported. JAK 2 and recombinant STAT 5 have been shown to form a stable complex *in vitro* by Flores-Morales *et al.* (35). These authors, however, maintain the necessity for JAK 2 phosphorylation for this interaction to occur. Our results, on the other hand, show no necessity for this and no evidence that phosphorylation of JAK 2 increases the amount of STAT 5 that coimmunoprecipitates. The results are instead consistent with the idea that JAK 2 can be either a kinase or an adapter molecule, as suggested previously by Fujitani *et al.* (36).

Because both STAT 5a and STAT 5b have been demonstrated to be involved in PRL signaling (37–40), we investigated the possibility that the aspartate mutant (through use of a second-receptor-associated kinase) would differentially activate one or the other STAT 5. The results show no distinction between the wild-type PRL and aspartate mutant in the activation of STAT 5a, but they do show a much reduced activation of STAT 5b in response to the aspartate mutant. This reduced activation of STAT 5b also results in reduced STAT 5a-STAT 5b heterodimers. Nonproliferative signaling, therefore, primarily activates STAT 5a. It will be of interest to investigate the actions of the aspartate mutant in tissues with well-defined nonproliferative responses to PRL.

PRL is normally secreted from the anterior pituitary as a combination of posttranslationally modified forms (17). In the rat, where the picture is not complicated by the presence of N-glycosylated forms (41), we have demonstrated the ratio of unmodified to phosphorylated PRL to vary reproducibly according to the stage of the estrus cycle and with pregnancy and pseudopregnancy (42, 43). Estrogen, for example, increases not only the total amount of PRL produced, but it also increases the proportion of it that is unmodified (42–45). Estrogen therefore increases the Nb2 proliferative activity of the PRL (21, 22). We hypothesize therefore that when a nonproliferative activity of PRL is desired, the amount of phosphorylated PRL increases. By working through an alternate kinase to JAK 2, phosphorylated PRL would not activate other JAK 2-connected signaling cascades in the cell, which normally collectively result in proliferation.

In summary, by using a nonproliferative form of PRL, which has nevertheless been demonstrated to signal in Nb2 cells, the results presented show a dissociation between JAK 2 and STAT 5 activation. Moreover, they support an important role for JAK 2 in proliferative responses and for STAT 5a in both proliferative and nonproliferative responses to PRL.

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