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Prolactin Activates Tyrosyl Phosphorylation of Insulin Receptor Substrate 1 and Phosphatidylinositol-3-OH Kinase*

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Prolactin (PRL) has been demonstrated to induce tyrosine phosphorylation and activation of the cytoplasmic tyrosine kinase JAK2. The present study represents an initial effort to identify the phosphorylation repertoire of the PRL receptor (PRLR). For this purpose we have modified the rat PRLR cDNA to encode an additional N-terminal epitope specifically designed to allow the rapid purification of the PRLR and associated proteins from transfected cells. The Flag-tagged PRLR was stably expressed in the human 293 cell line. PRL induced tyrosine phosphorylation of proteins of 85, 95, and 185 kDa from 10 to 30 min after PRL stimulation. Immunoblot analysis of immunoprecipitation indicates that p85 corresponds to the 85-kDa regulatory subunit of phosphatidylinositol (PI)-3′ kinase, p95 to PRLR, and p185 to insulin receptor substrate 1 (IRS-1). Both PI-3′ kinase and IRS-1 appear to associate with PRLR in a PRL-dependent manner. These results thus indicate that kinases other than JAK2, namely PI-3′ kinase, are activated by PRL.

PRL[1] binding to its cell surface receptor initiates a series of molecular interactions that ultimately determines the specific physiological response. Following PRL stimulation, PRL receptors are tyrosyl-phosphorylated; recent efforts to identify signal transducers activated by the PRL receptor have demonstrated that PRLR associates with and activates two cytoplasmic tyrosine kinases of the Janus T-K family (1–4), although JAK2 appears to be the major kinase involved in most responses.

A number of signaling molecules form stable complexes with other tyrosyl-phosphorylated receptors via an SH2 domain, including insulin receptor. The rationale for sharing common intracellular pathways between IR and PRLR is further substantiated by the insulin-like effect of growth hormone and to a lesser extent of PRL in a variety of cell types (5–7). These include 1) increased glucose-stimulated insulin secretion and decreased threshold of glucose stimulation (8), 2) increased insulin synthesis (9), 3) increased β-cell proliferation (10), and, more recently, it has been proposed that lactogenic hormones are primarily responsible for the enhanced islet function observed during pregnancy (11). To facilitate detection of the interaction repertoire and the phosphorylation repertoire of the PRLR, we have modified the rat PRLR cDNA to encode an additional N-terminal epitope specifically designed to allow the rapid purification of the PRLR and associated proteins from transfected cells. Our results clearly show that PRLR associates with insulin receptor substrate 1 and PI-3′ kinase. Upon PRL stimulation, both association with PRLR and tyrosyl phosphorylation of these two proteins are activated.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Ovine PRL was a gift from the National Hormone and Pituitary/NIDDK program (Baltimore, MD). The antiphosphotyrosine antibody (αPY), monoclonal IgG_2 bk antiserum to the 85-kDa subunit of PI-3′ kinase, and rabbit polyclonal antibody to IRS-1 (anti-rat C-terminal) were purchased from Upstate Biotechnology, Inc. Anti-Flag monoclonal antibody M2 and M1 affinity gel are products of IBI-Kodak.

Cells—The 293 fibroblast cells were grown in DMEM nut F12 medium containing 10% fetal calf serum. Several hours before transfection, cells were plated in a rich medium (two-thirds DMEM nut F12, one-third DMEM, 4.5 g/liter glucose) containing 10% fetal calf serum. Cells were incubated in the absence of serum overnight prior to hormone stimulation using serum-free DMEM/F12 (12).

Construction of the Flag PRLR (FPRLR) Expression Plasmid—The rat PRLR cDNA in the expression vector $pcDNA_3$ was modified to encode an additional Flag epitope, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys- between the signal peptide and the N terminus of the receptor via a recombinant polymerase chain reaction strategy (13). The Flag PRLR plasmid was used to transfect 293 cells. G-418 resistant cell lines overexpressing the FPRLR were selected for PRL binding. For purification studies, a clonal 293 FPRLR cell line was used.

Immunoprecipitation and Western Blotting—Confluent 293 cells, stably transfected with the Flag-tagged PRLR, were incubated in serum-free medium overnight. The cells were incubated at 37 °C, 5% CO_2 atmosphere. Cells were stimulated or not with oPRL (400 ng/ml) for 15 min. After stimulation, cells were rapidly washed with ice-cold phosphate-buffered saline and scraped in lysis buffer: 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM Na_3VO_4, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin A (10 μg/ml) on ice. Lysed cells were centrifuged at 14,000 rpm in a Sorvall centrifuge at 4 °C for 15 min. Lysates from control or stimulated cells were pooled, and 1.5 ml of supernatants were used for each immunoprecipitation. Immunocomplexes were collected using Protein A-Sepharose (Pharmacia Biotech. Inc.) and using the appropriate antibody. Incubation was carried out overnight at 4 °C. Samples were centrifuged and washed 3 times with lysis buffer, boiled 5 min in a mixture of 20% glycerol, 10% β-mercaptoethanol, 4.6% SDS, and 125 mM Tris, pH 6.8. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis on a 7.5% gradient polyacrylamide. Proteins were transferred on a polyvinylidene difluoride transfer membrane (Polyscreen™, DuPont NEN) using a semidry transfer cell (Trans-Blot SD, Bio-Rad). Blots were incubated with the appropriate antibody and visualized by ECL detection (Amersham). To reprobe the

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[1] The abbreviations used are: PRL, prolactin; PRLR, prolactin receptor; FPRLR, Flag prolactin receptor; oPRL, ovine prolactin; PI, phosphatidylinositol; IRS-1, insulin receptor substrate-1; GH, growth hormone; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium.

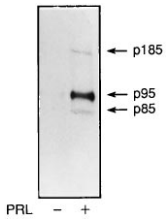

FIG. 1. **Copurification of proteins with FPRLR.** Tyrosine phosphoproteins in purified PRLR preparations revealed by antiphosphotyrosine immunoblot analysis. Confluent 293 FPRLR cells were incubated in the absence (−) or in the presence (+) of 400 ng/ml (18 nM) oPRL at 37 °C for 15 min prior to lysis and purification with anti-Flag column. The position (*arrows*) and molecular weights ($\times 10^{-3}$) of tyrosine phosphoproteins are indicated.

blot with another antibody, the blot was rinsed and incubated with stripping buffer (65 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol).

Copurification of PRLR Complexed Proteins Using Anti-Flag Column—Confluent 293 cells, stably transfected with the Flag-tagged PRLR, were incubated overnight in serum-free conditions and stimulated or not with oPRL (400 ng/ml). Cells were washed twice with ice-cold phosphate-buffered saline and scraped in lysis buffer (described above). After 30 min on ice, cell lysates were centrifuged at 42,000 rpm for 1 h at 4 °C. Supernatants were collected and diluted 1:5 with buffer B (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 3% (v/v) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM $CaCl_2$) and loaded on an Anti-Flag M1 affinity column (IBI-Kodak), washed with 30 ml of buffer B, and eluted with 6 ml of buffer C (Buffer B containing 2 mM EDTA without $CaCl_2$). Eluted samples were concentrated until an appropriate volume was obtained using Centriprep and Microcon tubes (Amicon) and boiled for 5 min in loading buffer (composition described above). Immunoblot analysis was performed as described previously (2).

In Vitro Phosphatidylinositol 3′-Kinase Activity—Immunocomplexes from 293-FPRLR cells immobilized on protein A-Sepharose were washed 3 times with lysis buffer, once with 0.5 M LiCl, 100 mM Tris-HCl, pH 7.4, and once with 100 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM EDTA. The PI-3′ kinase activity was assayed according to the method described by Auger *et al.* (14) in a final volume of a 50-μl reaction mixture containing 20 mM Hepes, pH 7.4, 2 mM sodium orthovanadate, 5 mM $MgCl_2$, 50 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 2 mg/ml phosphoinositides. The reaction was initiated by adding a $MgCl_2$-ATP mixture and was stopped after 20 min at 25 °C, by the addition of 100 μl of 1 M HCl. The lipids were extracted with 200 μl of chloroform/methanol (1:1), and they were resolved on thin layer chromatography TLC60 (Merck). The lipid phosphorylation was visualized by autoradiography.

RESULTS AND DISCUSSION

Understanding the PRL signaling pathway requires identification of the interaction repertoire and the phosphorylation repertoire of the PRLR. A recent report has suggested that GH, interferon-γ, and leukemia inhibitory factor stimulate tyrosyl phosphorylation of IRS-1 and its association with PI-3′ kinase (15) which provides a physiological basis for several of the insulin-like metabolic effects of GH. PRL receptor belongs to the cytokine superfamily of receptors and activates JAK2 in response to ligand binding; however, only limited numbers of reports are concerned with insulin-related effects of PRL. For example, the association between PRL and insulin-like growth factor has been described in several targets tissues (5, 6). To identify the PRLR interaction repertoire, we have designed a method using an epitope-modified PRLR and Ca^{2+}-dependent immunoaffinity chromatography to purify the PRLR and associated proteins. Analysis of the FPRLR proteins product in a transfected 293 cell line expressing the FPRL receptor was carried out. Binding experiments with ^{125}I-labeled PRL demonstrated 1×10^5 binding sites per 293 cell; the association constant ($K_a = 3\ nM^{-1}$) was similar to the wild type PRLR. The FPRLR was purified by an anti-Flag (M1) Sepharose chromatography column. Antiphosphotyrosine immunoblot of purified FPRLR treated without (−) or with (+) 18 nM oPRL (Fig. 1) revealed that tyrosyl phosphoproteins of 185 and 85 kDa specifically co-purified with PRL-treated FPRLR. The 95-kDa band representing the PRLR showed low basal levels of tyrosyl phosphorylation in the absence of PRL. A time course of stimulation by PRL (Fig. 2*A*) indicated that PRLRs are phosphorylated from 10 to 30 min after PRL stimulation, and no tyrosyl phosphorylation is observed at 60 min. In addition to the 95-kDa FPRLR, the immunoblot (Fig. 2*B*) with anti-Flag antibody revealed an 82-kDa product of C-terminal cleavage from PRLR which is present even in the absence of PRL. Interestingly, this product is not tyrosyl-phosphorylated (Fig. 2*A*). At 60 min, the PRLR is found in degradation product as a result of lysosomal degradation following internalization. In addition to the PRLR, a 185-kDa protein is tyrosyl-phosphorylated 20 min after stimulation, while the 85-kDa protein is activated 10 and 20 min after PRL stimulation. The identification of p85 as PI-3′ kinase was obtained after immunoprecipitation with anti PI-3′ kinase and immunoblot with antiphosphotyrosine (Fig. 2*C*); the same pattern of tyrosine phosphorylation upon PRL stimulation was obtained. Immunoblot analysis of purified FPRLR using immunoprecipitation with anti-Flag antibody revealed that p85 corresponds to the p85 subunit of PI-3′ kinase and that it displays a PRL-dependent association with FPRLR (Fig. 3*A*). Furthermore, p185 was shown to correspond to IRS-1 and was also associated with FPRLR in a PRL-dependent manner (Fig. 3*B*). The PRL-dependent association of both PI-3′ kinase and IRS-1 with FPRLR was obtained (Fig. 3, *A* and *B*, *lanes 3* and *4*) after immunoprecipitation with anti-Flag. PRL also induced the association of IRS-1 with PI-3′ kinase as shown by immunoprecipitation with anti-IRS-1 (Fig. 3*A*, *lanes 1* and *2*) or anti-PI-3′ kinase (Fig. 3*B*, *lanes 5* and *6*). Both of them were tyrosine-phosphorylated upon PRL stimulation (Fig. 3, *A* and *B*, *lanes 7* and *8*) following immunoblotting with anti-PI-3′ kinase (Fig. 3*A*) and anti-IRS-1 (Fig. 3*B*).

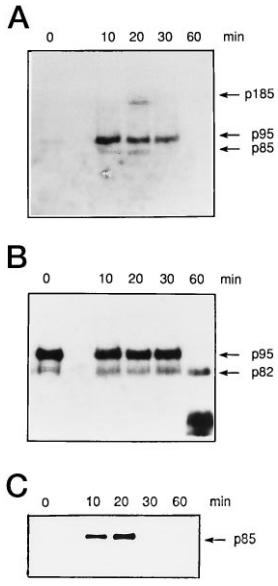

FIG. 2. **Time course of stimulation by PRL of tyrosine phosphorylation of proteins associated with FPRLR.** Confluent 293 FPRLR cells were incubated with 400 ng/ml (18 nM) oPRL at 37 °C for the times indicated prior to lysis and purification with anti-Flag column (*A* and *B*) or immunoprecipitation with αPI-3′ kinase (*C*). Purified proteins were immunoblotted with αPY (*A*), α-Flag (*B*), or αPY (*C*). The molecular weights ($\times 10^{-3}$) of migrating proteins are indicated.

293-FPRLR cells were maximally stimulated or not with oPRL (400 ng/ml), and FPRLR, IRS-1, and PI-3′ kinase were immunoprecipitated from these cells. The immunocomplexes were assayed for *in vitro* PI-3′ kinase activity. A representative experiment is shown in Fig. 4, where the presence of PI-3′

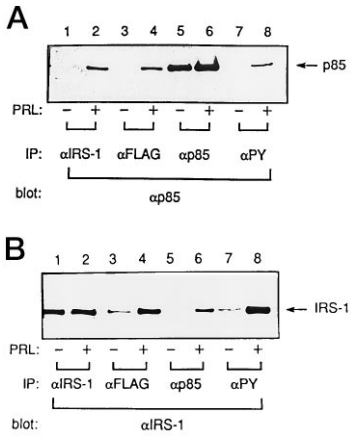

FIG. 3. **PRL activates association of the PRLR with IRS-1 and PI-3′ kinase.** Confluent 293 FPRLR cells were incubated in the presence (+) or absence (−) of 400 ng/ml (18 nM) oPRL at 37 °C for 15 min. Whole cell lysates were immunoprecipitated with αIRS-1 (*lanes 1* and *2*), α-Flag (*lanes 3* and *4*), αPI-3′ kinase (*lanes 5* and *6*), and αPY (*lanes 7* and *8*). Immunoprecipitated proteins (*IP*) were immunoblotted with αPI-3′ kinase (*A*) or αIRS-1 (*B*). The positions of the 85-kDa subunit of PI-3′ kinase (p85) and of IRS-1 (p185) are indicated.

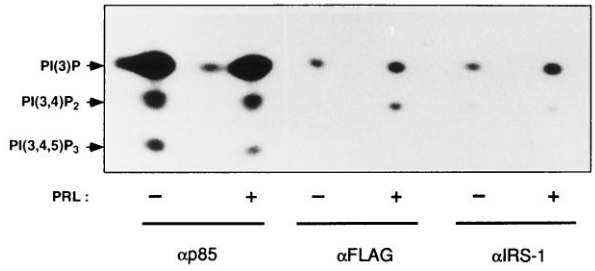

FIG. 4. **PI-3′ kinase activity associates with PRLR and IRS-1 in a PRL-dependent manner.** 293-FPRLR cells (2×10^7 cells) were stimulated or not with oPRL (400 ng/ml) for 20 min. Cell lysates were subjected to immunoprecipitation with antibodies anti-IRS-1, anti-Flag, and anti-PI-3′ kinase, as indicated. The PI-3′ kinase activity was assayed in the presence of a mixture of phosphoinositides (PI, PI(4)P, $PI(4,5)P_2$) and [γ-^{32}P]ATP. The products of the reaction were extracted with chloroform/methanol and resolved by thin layer chromatography. The figure shows a representative autoradiogram where the migration of PI(3), $PI(3,4)P_2$, and $PI(3,4,5)P_3$ is indicated.

kinase activity in the immunocomplexes of FPRLR and IRS-1 was evidenced by the presence of PI(3)P and $PI(3,4)P_2$ labeled with radioactive phosphate. The PI-3′ kinase activity was significantly increased when cells were stimulated with PRL compared with nonstimulated. This increase in PI-3′ kinase activity seems to be due to a higher amount of PI-3′ kinase associated to PRLR induced by PRL, since the total PI-3′ kinase immunoprecipitated activity was not modified. These data fit well with Western blot results (Fig. 3*A*), where PRL stimulation results in PI-3′ kinase association to PRL receptor.

A major pathway for signal transduction has been described for the PRLR, implicating the JAK2 protein kinase. Ligand binding to PRLR activates this tyrosine kinase (2, 4) which appears to be constitutively associated with the receptor. A cytoplasmic proximal region of the receptor is required for JAK2 association, more precisely the Box 1 and the adjacent residues upstream of Box 2 (12). Multiple members of the cytokine receptor family can activate JAK2; some have also been shown to stimulate tyrosyl phosphorylation of IRS-1 (15–17). For example, GHR and IL9-R associated JAK2 are able to tyrosine-phosphorylate IRS-1 upon ligand binding (15, 17). In this report, we describe a PRL-dependent association with FPRLR and tyrosine phosphorylation of IRS-1, albeit the type of interaction between JAK2 and IRS-1 after PRL induction is unknown. A possible direct association would make JAK2 an obvious candidate for the tyrosine kinase responsible for the PRL-dependent tyrosine phosphorylation of IRS-1. The possibility of an auxiliary molecule common to the cytokine receptor family that could bind JAK2 and induce phosphorylation of IRS-1 cannot be excluded. IRS-1 may interact directly or indirectly with the PRLR; cytoplasmic regions involved in this association have to be determined. In the case of GHR (15), none of the tyrosines of the cytoplasmic domain appear to be necessary for IRS-1 tyrosyl phosphorylation indicating that IRS-1 interacts with other as yet unidentified proteins. In conclusion, it appears that signaling through IRS-1 may be common to multiple members of this family that activate JAK2.

IRS-1 has been implicated as intermediate between insulin receptor and several signal molecules. It provides binding sites for SH2 domains of PI-3′ kinase subunit p85. In our study, we detected an association of these two molecules together and with the Flag PRLR. Activation of this enzyme can lead to proliferation and regulation of the cell cycle, glucose uptake, and vesicular trafficking of proteins (18). The signaling pathway involving PI-3′ kinase could explain the insulin-related effects of PRL. Further experiments are necessary to determine tyrosine-phosphorylated regions implicated in the interaction of the SH2 domains of p85 and either the activated PRLR or a phosphorylated protein intermediate after stimulation by PRL. The membrane proximal domain of PRLR contains a YSMM sequence which is a putative binding site for the SH2 domain of p85 (19).

These experiments revealed a new repertoire of signaling molecules of the PRLR, involving different pathways. Interconnection between these pathways has to be defined more precisely.

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REFERENCES

1. Rui, H., Kirken, R. A., and Farrar, W. (1994) *J. Biol. Chem.* **269,** 5364–5368
2. Lebrun, J. J., Ali, S., Sofer, L., Ullrich, A., and Kelly, P. A. (1994) *J. Biol. Chem.* **269,** 14021–14026
3. Dusanter-Fourt, I., Muller, O., Ziemiecki, A., Mayeux, P., Drucker, B., Djiane, J., Wilks, A., Harpur, A. G., Fischer, S., and Gisselbrecht, S. (1994) *EMBO J.* **13,** 2583–2591
4. Campbell, G. S., Argetsinger, L. S., Ihle, J. N., Kelly, P. A., Rillema, J. A., and Carter-Su, C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5232–5236
5. Nicoll, C. S., Anderson, T. R., Hebert, N. J., and Russell, S. M. (1985) in *Prolactin Basic and Clinical Correlates* (MacLeod, R. M., Thorner, M. O., and Scapagnini, U., eds) pp. 393–413, Fidia Research Series, Liviana Press, Padua
6. Jahn, G. A., Diolez-Bojda, F., Belair, L., Kerdelhué, B., Kelly, P. A., Djiane, J., and Edery, M. (1991) *Biomed. Pharmacother.* **45,** 15–23
7. Billestrup, N., Moldrup, A., Serup, P., Mathews, L. S., Norstedt, G., and Nielsen, J. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7210–7214
8. Sorenson, R. L., Brelje, T. C., Hegre, O. D., Marshall, S., Anaya, P., and Sheridan, J. (1987) *Endocrinology* **121,** 1447–1453
9. Brelje, T. C., Halaire, P., Hegre, O., and Sorenson, R. L. (1989) *Endocrinology* **125,** 2392–2399
10. Brelje, T. C., and Sorenson, R. L. (1991) *Endocrinology* **128,** 45–57
11. Sorenson, R. L., and Stout, L. E. (1995) *Endocrinology* **136,** 4092–4098
12. Lebrun, J. J., Ali, S., Ullrich, A., and Kelly, P. A. (1995) *J. Biol. Chem.* **270,** 10664–10670
13. Zhang, W., Johnson, J. D., and Rutter, W. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11317–11321
14. Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989) *Cell* **57,** 167–175
15. Argetsinger, L. S., Hsu, G. W., Myers, M. G., Billestrup, N., White, M. F., and Carter-Su, C. (1995) *J. Biol. Chem.* **270,** 14685–14692
16. Keegan, A. D., Nelms, K., White, M., Wang, L-M., Pierce, J. H., and Paul, W. E. (1994) *Cell* **76,** 811–820
17. Yin, T., Keller, S. R., Quelle, F. W., Witthuhn, B. A., Tsang, M. L.-S., Lienhard, G. E., Ihle, J. N., and Yang, Y.-C. (1995) *J. Biol. Chem.* **270,** 20497–20502
18. Schu, P. V., Takegawa, K., Fay, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) *Science* **260,** 88–91
19. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302

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