ELSEVIER

Biochimica et Biophysica Acta 1497 (2000) 89–93

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.elsevier.com/locate/bba

Prolactin stimulation of tyrosyl phosphorylation of Shc proteins in Nb_2 lymphoma cells, but not mammary tissues

T.X. Yu, J.A. Rillema *

Department of Physiology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201, USA

Received 18 November 1999; received in revised form 31 January 2000; accepted 21 February 2000

Abstract

Prolactin (PRL) stimulates lactogenesis in mammary cells and mitogenesis in a variety of cell types including Nb_2 cells. Studies indicate that a different composite of signaling pathways is involved in the PRL stimulation of mitogenesis as compared to lactogenesis. In the present studies, PRL is shown to stimulate the tyrosyl phosphorylation of all three isoforms of Shc proteins in Nb_2 cells (mitogenesis), but not in the mammary gland. Maximal phosphorylation of the Shc proteins is expressed between 10 and 15 min after a 50-ng/ml PRL treatment. In addition, there is an increased association between the Grb2 protein and Shc proteins upon PRL stimulation. However, no increased association between JAK2 and Shc proteins was observed in either the Nb_2 cells or mammary tissues.

Keywords: Prolactin; Nb_2 cell; Mouse mammary gland; Shc protein; Tyrosyl phosphorylation

1. Introduction

Prolactin (PRL) is a peptide hormone with a wide range of functions including regulation of differentiative and developmental processes in the mammary gland and certain male accessory sex glands, and the regulation of specific immune responses [1]. The PRL receptor (PRLR) has three forms (long, intermediate, and short), which differ primarily in the length of their cytoplasmic domains [2]. These PRLR isoforms belong to the cytokine/hematopoietin receptor superfamily. This superfamily also includes receptors for growth hormone (GH) and a variety of cytokine and growth factors [3,4]. Comparison of the amino acid sequences of the PRLR and GHR reveals a linear arrangement of regions of similar sequences [5], and both receptors are evolved from a single gene localized in the same region of human chromosome 5 [6]. They have structural and functional similarities. It has also become apparent that they recruit and/or activate many of the same kinases and signaling proteins in their signal transduction pathways. Some of the most common signaling molecules involved in both systems include the Janus kinase (JAK2) [7–11], the MAP kinases (MAP) [12–15] and the Stat proteins (Stat 5) [16–19].

Shc proteins have been shown to be involved in the GH signaling pathway [20,21]. Upon GH activation, Shc proteins are phosphorylated on tyrosyl residues. The activated Shc acts as a linker protein and binds to other signaling proteins via its SH2 (src homology) domains, particularly to Grb2. However, involvement in PRL-induced mitogenesis and lactogenesis has not heretofore been determined. In order

* Corresponding author. Fax: +1-313-577-5494; E-mail: jrillema@med.wayne.edu

0167-4889/00/$ – see front matter
PII: S0167-4889(00)00035-5

to study the possible involvement of Shc proteins in PRL-induced processes, two primary model systems were employed: Nb2 cells in which the mitogenic effect of PRL is expressed, and the mouse mammary gland explant system in which the lactogenic effects of PRL are expressed. The Nb_2 cell line, the only cell line known to express the intermediate form of the PRLR [22], is dependent on lactogenic hormone stimulation for continuous progression through the cell cycle [23]. The goals of the present studies are not only to examine the involvement of the Shc proteins in PRL signal transductions in both Nb_2 cells and mouse mammary gland, but also to study the possible relationship between the Shc proteins and other proteins. Since the involvement of Shc in the tyrosine kinase signaling pathways requires its phosphorylation, we isolated Shc proteins from Nb_2 and mouse mammary gland tissues to detect its tyrosyl phosphorylation states. In further studies, we investigated the time and concentration effects of PRL on Shc protein phosphorylations.

2. Materials and methods

2.1. Nb_2 cell preparation

Nb_2 cells (provided by Dr. C.T. Beer) were grown in 75-mm^2 culture flasks containing Fisher's medium supplemented with 10% fetal calf serum, 10% horse serum (Sigma, St. Louis, MO), 1×10^{-4} M 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin (Eli Lilly, Indianapolis, IN), 7.5% $NaHCO_3$, and 290 mg/ml glutamine. After cells have entered the log growth stage they were collected by centrifugation at $200\times g$. Cells were arrested at G0 by incubation for 24 h in 0% Fisher's (Fisher's having no HS and FCS) at 500 000 cells/ml. The Nb_2 cells were then collected by centrifugation at $200\times g$ for 5 min and resuspended in fresh Fisher's medium at 1 million cells/ml. Cell aliquots were treated with different concentrations of oPRL (NIH-P-S-17, a gift from NIDDK) for different times. Treated cell samples were then collected by centrifugation. To individual samples were added lysis buffer at $2–10\times10^7$ cells/ml according to different experiments. The total lysates were used for immunoprecipitation and Western blotting assays.

2.2. Mouse mammary gland explant preparation

Twelve- to 14-day pregnant mice were killed by cervical dislocation after which caudal and inguinal pairs of mammary glands were removed and placed in Hank's balanced salt solution. The explants were prepared as described by Rillema [24]. Glands were cut into explants of approximately 3 mg each. Explants from 18 to 20 animals were randomly placed in 60×15 mm culture dishes containing siliconized lens paper floating on 6 ml of M-199 medium. This medium was supplemented with 10^{-7} M cortisol and 1 μg/ml insulin. Tissues were incubated for 24 h at 37°C in an atmosphere of 95% O_2–5% CO_2. Then the medium was changed with M-199 without any hormone for 24 h. Ovine PRL at a concentration of 1 μg/ml were added and tissues were cultured for variable time periods. Tissues were homogenized in 2 times lysis buffer which contained 2% NP-40, 50 mM NaCl, 10 mM Tris, 30 mM $Na_2P_2O_7$, 2.5 mM EDTA, 1 mM $NaVO_4$, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin, and then centrifuged at $100\,000\times g$ for 1 h at 4°C. Soluble proteins in lysis preparations were subject to immunoprecipitation and Western blotting assays.

2.3. Immunoprecipitation and immunoblotting

Nb_2 cell or MMG lysates were immunoprecipitated with anti-SHC polyclonal antibody (UBI #06-203, 5 μg/ml) at 4°C overnight. SHC–antibody immunocomplexes were collected by incubation with 50 μl anti-rabbit IgG agarose (Sigma, A-1027) for 4 h at 4°C. Proteins were solubilized in 50 μl SDS–Laemmli sample buffer and heated 95°C for 5 min. Samples were separated by SDS–PAGE (8–20% linear gradient) under reducing conditions and transferred to PVDF membranes (Schleicher and Schuell). Membranes were probed with anti-phosphotyrosine (UBI, #05-321 50 ml at 1:1000), anti-SHC (UBI, #06-203; 50 ml at 1:2000), anti-GRB2 (UBI, #05-372; 20 ml at 1:1000) or anti-JAK2 (UBI, #06-255; 20 ml 1:2000) for 2 h. The secondary antibodies used were anti-rabbit IgG–HRP conjugate (Amersham NA 934; 1:3000 to 1:5000) or anti-mouse IgG HRP conjugate (Amersham NA 931 at 1:5000) according to different primary antibodies for 1.5 h. Detection was accomplished by incubation with en-

hanced chemiluminescence reagents (Amersham) and exposure to X-ray film.

3. Results

In initial experiments, the time course of tyrosyl phosphorylations of Shc proteins was determined. Nb_2 cells arrested in G0/G1 were incubated in the absence or presence of PRL for different periods of time. Cells were lysed and the Shc proteins were immunoprecipitated with anti-Shc antibody serum. The isolated proteins were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were probed with either anti-phosphotyrosine (Fig. 1) or anti-Shc (Fig. 2a) antibodies to examine the degree of Shc tyrosyl phosphorylation in response to PRL. As is shown in Fig. 1, three distinct tyrosyl phosphorylated bands were apparent; these migrated at 46, 52 and 66 kDa. These bands are consistent with the molecular weights of the three isoforms of the Shc proteins. They were phosphorylated rapidly within 5 min, maximally phosphorylated between 10 and 15 min and then dephosphorylated. Fig. 2b shows that there was also an association between Shc and Grb2, since Grb2 was visualized when the same membranes were immunoblotted with anti-Grb2 antibody.

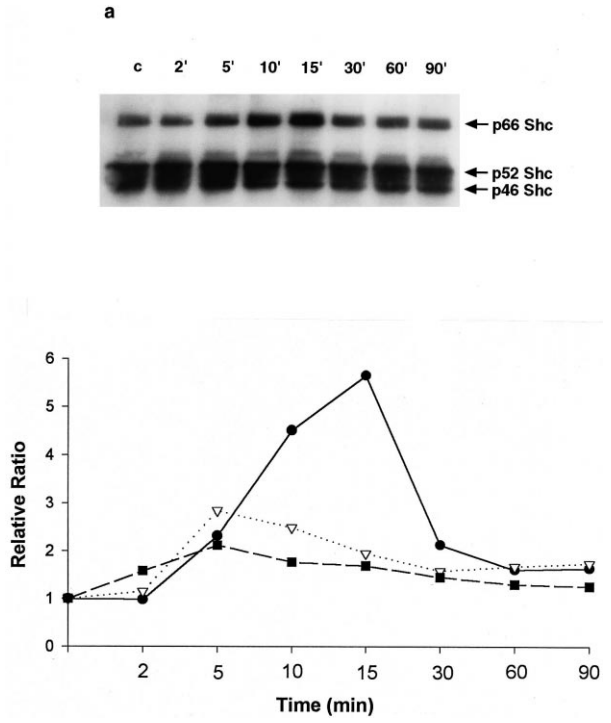

Fig. 1. Time-course study of PRL-induced tyrosyl phosphorylation of Shc proteins in Nb_2 cells. G1-arrested Nb_2 cells (1×10^8/ml) were incubated in the absence or presence of oPRL (50 ng/ml) for the indicated time (min). Total lysate preparations were immunoprecipitated with anti-Shc polyclonal antibody. Isolated proteins were separated by 8–20% SDS–PAGE. The transferred PVDF membrane was probed with anti-phosphotyrosine antibody (top panel). The phosphorylations of Shc proteins were quantitated using a scanning laser densitometer (bottom panel). ●, p66 band; ▽, p52 band; ■, p46 band.

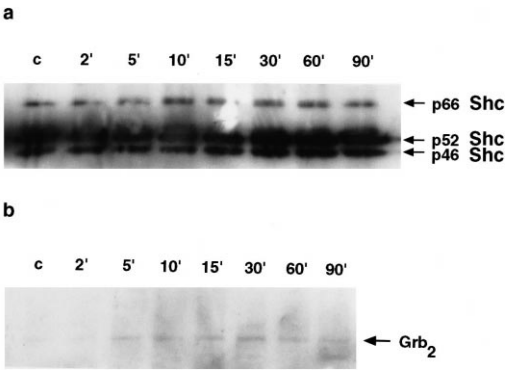

Fig. 2. Time course of Shc protein phosphorylations in response to PRL. The same membrane used in Fig. 1 was stripped and re-probed with anti-Shc (a) or anti-Grb2 (b) antibodies.

In further experiments, Shc protein phosphorylation was studied in Nb_2 cells treated with increas-

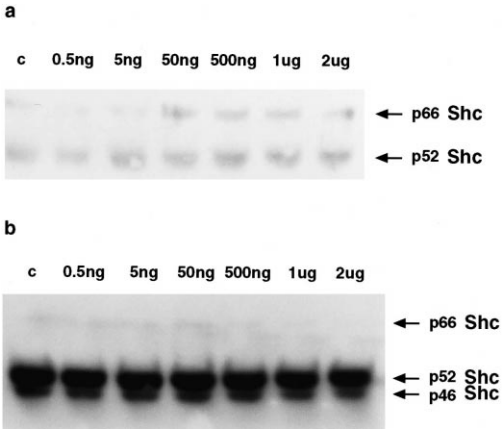

Fig. 3. Effect of PRL concentrations on tyrosyl phosphorylation of the Shc protein. G1-arrested Nb_2 cells (2×10^7/ml) were treated with increasing concentrations of oPRL for 10 min. Total lysate preparations were immunoprecipitated with anti-Shc polyclonal antibody. Isolated proteins were separated by SDS–PAGE. The transferred membrane was immunoblotted with anti-phosphotyrosine (a) or anti-Shc (b) antibodies.

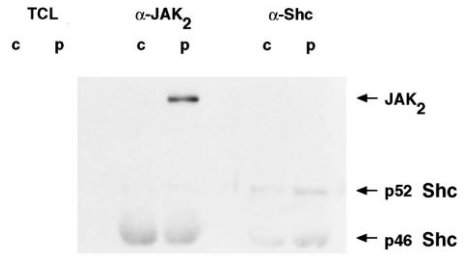

Fig. 4. Effect of PRL on tyrosyl phosphorylations of the JAK2 kinase and Shc protein in Nb_2 cells. G1 arrested Nb_2 cells (2×10^7/ml) were incubated in the absence or presence of oPRL (50 ng/ml) for 10 min. Total cell lysate preparations (TCL) were immunoprecipitated with anti-JAK2 or anti-Shc antibodies. Samples were separated by SDS–PAGE and transferred to a PVDF membrane, which was probed with anti-phosphotyrosine antibody.

ing PRL concentrations from 500 pg/ml to 2 μg/ml. As shown in Fig. 3, 50 ng/ml PRL significantly increases phosphorylation of two Shc proteins (the 52- and 66-kDa isoforms). Similar to earlier experiments, PRL is also shown to stimulate JAK2 kinase activity. In order to determine the effect of PRL on phosphorylation and possible interaction of JAK2 and Shc proteins, control or PRL-treated cell extracts were immunoprecipitated with anti-JAK2 or anti-Shc antibodies, and then immunoblotted with anti-phosphotyrosine antibody. PRL increased both JAK2 and Shc tyrosyl phosphorylations (Fig. 4). In order to determine whether there is any association between JAK2 and Shc proteins, the same membranes were immunoblotted with both anti-JAK2 and anti-Shc antibodies. There was no detectable association between JAK2 and Shc in PRL-treated samples (Fig. 5).

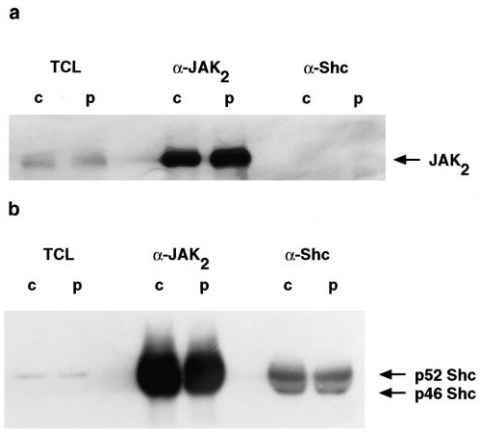

Fig. 5. The same membrane used in Fig. 4 was stripped and reprobed with anti-JAK2 (a) or anti-Shc (b).

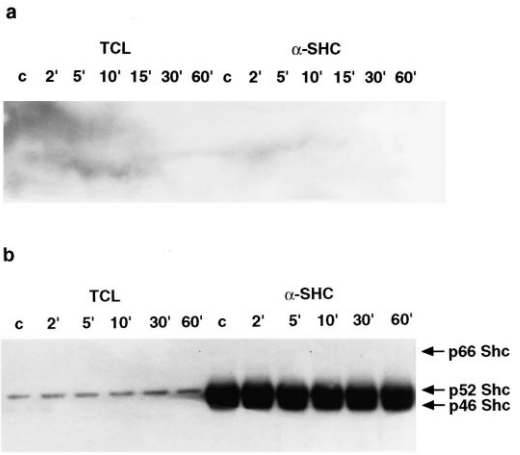

Fig. 6. Time-course of the effect of PRL on Shc proteins in mouse mammary gland tissues. The explants were cultured for 24 h with M-199 in the presence of 1 μg/ml insulin and 10^{-7} M cortisol followed by another 24 h with M-199 without insulin and cortisol. After pre-incubation, 1 μg/ml PRL was added for different periods of time and Shc proteins were immunoprecipitated from total tissue lysate (TCL). The separated samples were transferred to a PVDF membrane and probed with either anti-phosphotyrosine (a) or anti-Shc (b) antibodies.

In a subsequent series of experiments, the possible involvement of Shc proteins in PRL signaling transduction was investigated in mouse mammary gland explants. The cultured mouse mammary gland tissues were treated with or without PRL (1 μg/ml) and total tissue lysate was immunoprecipitated with anti-Shc antibody. Isolated proteins were separated by SDS–PAGE. Fig. 6 shows that there was no significant tyrosyl phosphorylation in either control or PRL-treated samples after immunoblotting with anti-phosphotyrosine antibody. In order to confirm the existence of Shc proteins in these samples, a subsequent probing of the same membrane was done with anti-Shc antibodies. As predicted, the same amount of Shc proteins were visualized in both control and PRL-treated groups.

4. Discussion

As mentioned above, the roles of Janus kinase and MAP kinases have been well studied in signaling pathways of the cytokine/hematopoietin receptor superfamily. It is well established that the Shc pro-

teins play a role as an adapter protein in many of these signaling pathways. However, the involvement of the Shc protein in the PRL signaling pathway has not been well studied.

The time-course experiments showed that an increased tyrosyl phosphorylation level was observed in the PRL-treated samples from Nb_2 cells. In addition, all three isoforms of Shc proteins were phosphorylated. In dose–response experiments, it was found that 50 ng/ml PRL could maximally stimulate tyrosyl phosphorylation of Shc proteins. However, compared with that of JAK2, the degree of tyrosyl phosphorylation of Shc proteins in response to PRL was less dramatic.

Although Shc is believed to be a substrate for JAK2 in T47D cells, a human breast cancer cell line [25], we were unable to detect the association between JAK2 and Shc upon PRL stimulation in Nb_2 cells. These results raise the possibility that other proteins or adapter molecules might be involved in mediating or enhancing the association of JAK2 with Shc.

Although the involvement of Shc in the tyrosine kinase signaling pathways appears to require its phosphorylation in Nb_2 cells, no significant tyrosyl phosphorylation in either the control or PRL-treated mouse mammary gland tissues was observed. Possible explanations for this phenomenon are: (1) the concentration of Shc proteins in mouse mammary gland tissues is very low; (2) the major functional isoform of Shc proteins is different in Nb_2 cells from that in the mouse mammary gland; (3) full activation of Shc proteins may require synergism with other growth factors; or (4) Shc proteins may not be involved in the lactogenic actions of PRL. The latter possibility appears most plausible at present.

References

[1] N.D. Horseman, Endocrinology 135 (1994) 1289–1291.
[2] J.M. Boutin, C. Jolicoeur, H. Okamura, J. Gagnon, M. Edery, M. Shirota, D. Banville, I. Dusanter-Fourt, J. Djiane, P.A. Kelly, Cell 53 (1988) 69–77.
[3] J.F. Bazan, Immunol. Today 11 (1990) 350–354.
[4] D. Cosman, S.D. Lyman, R.L. Idzerda, M.P. Beckmann, L.S. Park, R.G. Goodwin, C.J. March, Trends Biochem. Sci. 15 (1990) 265–270.
[5] V. Goffin, P.A. Kelly, Clin. Mol. Endocrinol. 45 (1996) 247–255.
[6] K.C. Arden, J.M. Boutin, J. Djiane, P.A. Kelly, W.K. Cavenee, Cytogenet. Cell Genet. 53 (1990) 161–165.
[7] H. Rui, R.A. Kirken, W.L. Farrar, J. Biol. Chem. 269 (1994) 5364–5368.
[8] G.S. Campbell, L.S. Argetsinger, J.N. Ihle, P.A. Kelly, J.A. Rillema, C. Carter-Su, Proc. Natl. Acad. Sci. USA 91 (1994) 5232–5236.
[9] J.J. Lebrun, S. Ali, L. Sofer, A. Ullrich, P.A. Kelly, J. Biol. Chem. 269 (1994) 14021–14026.
[10] L.S. Argetsinger, G.S. Campbell, X. Yang, B.A. Witthuhn, O. Silvennoinen, J.N. Ihle, C. Carter-Su, Cell 74 (1993) 237–244.
[11] L.S. Smith, D.J. Meyer, N. Billestrup, G. Norstedt, J. Schwartz, C. Carter-Su, Mol. Endocrinol. 10 (1996) 519–533.
[12] A.R. Buckley, Y.P. Rao, D.J. Buckley, P.W. Gout, Biochem. Biophys. Res. Commun. 204 (1994) 1158–1165.
[13] I.G. Camarillo, B.E. Linebaugh, J.A. Rillema, Proc. Soc. Exp. Biol. Med. 215 (1997) 198–202.
[14] G.S. Campbell, L. Pang, T. Miyasaka, A.R. Saltiel, C. Carter-Su, J. Biol. Chem. 267 (1992) 6074–6080.
[15] C. Moller, A. Hansson, B. Enberg, P.E. Lobie, G. Norstedt, J. Biol. Chem. 267 (1992) 23403–23408.
[16] F. Gouilleux, H. Wakao, M. Mundt, B. Groner, EMBO J. 13 (1994) 4361–4369.
[17] N. Tourkine, C. Schindler, M. Larose, L.M. Houdebine, J. Biol. Chem. 270 (1995) 20952–20961.
[18] T.J. Wood, D. Sliva, P.E. Lobie, T.L. Pircher, F. Gouilleux, H. Wakao, J.A. Gustafsson, B. Groner, G. Norstedt, L.A. Haldosen, J. Biol. Chem. 270 (1995) 9448–9453.
[19] F. Gouilleux, C. Pallard, I. Dusanter-Fourt, H. Wakao, L.A. Haldosen, G. Norstedt, D. Levy, B. Groner, EMBO J. 14 (1995) 2005–2013.
[20] J. Vanderkuur, A. Allevanna, N. Billestrup, G. Norstedt, C. Carter-Su, J. Biol. Chem. 270 (1995) 7587–7593.
[21] A. Thirone, C. Carvalho, M. Saad, Endocrinology 140 (1998) 55–62.
[22] S. Ali, I. Pellegrini, P.A. Kelly, J. Biol. Chem. 266 (1991) 20110–20117.
[23] P.W. Gout, R.L. Noble, C.T. Beer, Biochem. Cell Biol. 64 (1985) 659–666.
[24] J.A. Rillema, Endocrinology 92 (1973) 1673–1679.
[25] R. Das, B.K. Vonderhaar, Oncogene 13 (1996) 1139–1145.