Volume 300, number 1, 93–96 FEBS 10856 March 1992

A cytosolic protein tyrosine kinase in rat adipocytes

Assia Shisheva and Yoram Shechter*

Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 28 January 1992

Previous studies suggested that insulin receptor tyrosine kinase (IRTK) is the sole tyrosine kinase in rat adipocytes. We now report that this cell type also contains a cytosolic soluble protein tyrosine kinase (CytPTK) which is not related to IRTK. The enzyme phosphorylated PolyGlu$_4$Tyr whith high efficiency at a rate of 20 ± 2 pmol PTyr/20 μg PolyGlu$_4$ Tyr/20 min/μg cytosolic protein. Upon gel filtration chromatography the enzyme activity was eluted as a single peak corresponding to a molecular mass of 53 ± 3 kDa. Unlike IRTK, CytPTK activity was supported by Co^{2+} rather than by Mn^{2+}, and it was not inactivated by *N*-ethylmaleimide. The enzyme was extremely sensitive to inhibition by staurosporine (ID_{50} = 3 nM) as opposed to IRTK (ID_{50} = 8 μM). In addition, CytPTK (but not IRTK) was largely activated by vanadate ions. Agents which affect the serine/threonine phosphorylation state of cell proteins did not alter CytPTK activity when subjected to intact adepocytes. In a cell-free system CytPTK activity was largely reduced by pretreatment with immobilized alkaline phosphatase at physiological pH. The possibility that CytPTK participates in insulin-independent regulation of glucose metabolism is suggested.

Protein tyrosine kinase; Glucose metabolism; Rat adipocytes

1. INTRODUCTION

The level of protein phosphorylation on tyrosyl residues is extremely low compared to phosphorylation on seryl and threonyl residues in intact cellular systems [1]. The amount of phosphotyrosine in cells comprises less than 0.1% of the combined amounts of phosphoserine and phosphothreonine [1]. Nevertheless, tyrosine-specific protein kinases (E.C.2.7.11) have attracted much attention in recent years for two reasons: first, they are an intrinsic entity of the receptors for insulin and most growth factors [2], and secondly, they are transcription products of several oncogenes [3]. Recently, tyrosine kinase activities have been identified in several normal tissues and cells, including spleen, liver, lung, thymus, brain, platelets and erythrocytes [4–7]. Since they are devoid of ligand bnding ability, they are referred to as 'nonreceptor' PTKs. These kinases are present in particulate as well as in cytosolic fractions and possess molecular masses varying from 40 to 120 kDa [7]. Due to their low abundance in normal cells, nonreceptor-associated PTKs have not been extensively studied. Their modes of participating in cellular metabolism are not known, neither are their natural substrates.

In mammalian adipocytes nonreceptor tyrosine kinases have not been described so far. In this study we report the presence of a novel cytosolic PTK in rat adipocytes. The enzyme was partially purified and characterized. Its possible role in regulating glucose metabolism in adipocytes is discussed.

2. MATERIALS AND METHODS

2.1. *Preparation of rat adipocytes and high-speed supernatant fraction*

Rad adipocytes were prepared from fat pads of male Wistar rats (180–250 g) by treatment with collagenase [8]. Cytosolic cell extract was obtained by homogenizing the cells in 50 mM HEPES, pH 7.4, supplemented with 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin and 5 μg/ml aprotinin, and centrifuging thereafter at 40,000 × *g* for 60 min. Where indicated, the adipocytes were pretreated with $NaVO_3$, H_2O_2, okadaic acid, bt_2-cAMP, isoproterenol, TPA for 40 min at 37°C, or for 5 min with insulin. Cell suspensions were then supplemented with equal volumes of 50 mM HEPES, pH 7.4, containing 2 mM $NaVO_3$, followed by lysing of adipocytes. The crude cell extracts were collected by centrifuging the cell homogenates through a silicone oil layer [9]. Protein concentrations [10] were normalized and tyrosine kinase activity was determined in aliquots.

2.2. *Tyrosine kinase activity measurements*

Unless otherwise indicated, the standard enzyme assay mixture (final volume 60 μl in 50 mM HEPES, pH 7.4) contained the enzyme source (crude extracts, fractions after column chromatography or purified enzyme), 10 mM $MgCl_2$, 2 mM cobalt (II) acetate and 100 μM ATP. Following 30 min preincubation, the reaction was initiated by adding PolyGlu$_4$Tyr (final concentration, 0.67 mg/ml), proceeded for 20 min at 22°C and was terminated by adding EDTA (20 mM) and

* Yoram Shechter is the incumbent of C.H. Hollenberg Chair in Metabolic and Diabetes Research, established by the Friends and Associates of Dr. C.H. Hollenberg of Toronto, Canada.

Abbreviations: WGA, wheat germ agglutinin; PTK, protein tyrosine kinase; IRTK, insulin receptor tyrosine kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; TPA, 12-tetradecanoylphorbol-13-acetate; bt_2cAMP, N^6, 2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate; PolyGlu$_4$Tyr, poly-[(Glu Na:Tyr) 4:1)]; PTyr, phosphotyrosine.

Correspondence address: Y. Shechter, Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel. Fax: (972) (8) 344116.

vanadate (1 mM). Basically the same procedure was applied when WGA-agarose purified IRTK was used except that $MnCl_2$ (2 mM), was substituted for Co^{2+}, and insulin (10^{-7} M) and Triton X-100 (0.1%) were present during the reaction. The phosphotyrosine content of $PolyGlu_4Tyr$ was quantitated by RIA, using antibodies to phosphotyrosine [11].

2.3. Purification procedures

Partially purified insulin receptor was prepared from rat liver by solubilization of plasma membrane fraction with Triton x-100, adsorption to WGA-agarose affinity column and elution with *N*-acetyl-D-glucosamine as described [12]. Partial purification of CytPTK activity was achieved by chromatography of the crude adipocytic extract (12 ml, ~10 mg cytosolic protein) on a column of Cibacron blue 3GA-agarose. The column (2 × 1 cm) was pre-equilibrated and washed with 50 mM HEPES, pH 7.4, and eluted with the same buffer, containing 0.5 M NaCl. The eluted proteins were dialyzed, lyophilized, and reconstituted in 50 mM HEPES, pH 7.4. The material was loaded onto a Sephadex G-200 column (1.3 × 60 cm), which was pre-equilibrated and eluted with 50 mM HEPES, pH 7.4, at 7°C (flow rate 4 ml/h). Tyrosine kinase activity was determined in the aliquots of collected fractions (0.5 ml).

The data on figures and tables are presented as the means ± S.E.M. ($n \geq 3$).

3. RESULTS AND DISCUSSION

3.1. *Identification of CytPTK activity in rat adipocytes*

Freshly prepared rat adipocyte suspension was supported with proteases' inhibitors and lysed in the absence of detergents. The 40,000 × *g* supernatant was initially examined for its $PolyGlu_4Tyr$-phosphorylating activity under the standard assay conditions applied for IRTK [13] namely in the presence of 2 mM Mn^{2+}, and 10 mM Mg^{2+}. Under these conditions little phosphorylating activity was observed (Table I). However, when Co^{2+} was substituted for Mn^{2+}, the extent of phosphorylation increased largely. Following large scale characterization, the optimal divalent ions requirement was found to be Co^{2+} (1–2 mM) plus Mg^{2+} (10–15 mM); Mn^{2+}, Ca^{2+} and Zn^{2+} did not support phosphorylation. ATP was the preferred phosphoryl donor, although UTP and GTP could also be utilized to some extent (Table I).

Table I

Phosphorylation of $PolyGlu_4Tyr$ by CytPTK in the presence of various divalent cations and nucleotides

Reaction conditions	CytPTK activity pmol PTyr/20 μg $PolyGlu_4Tyr$/20 min	Relative potency %
Divalent ions[a]		
None	0 ± 0.01	0
Mg^{2+}, 10 mM	8 ± 0.5	15
Mn^{2+}, 2 mM	0.03 ± 0.004	0
Mg^{2+}, 10 mM + Mn^{2+}, 2 mM	8.00 ± 0.03	15
Co^{2+}, 2 mM	17 ± 1.3	31
Mg^{2+}, 10 mM + Co^{2+}, 2 mM	54 ± 3	100
Ca^{2+}, 2 mM	0.04 ± 0.007	0
Zn^{2+}, 2 mM	0.02 ± 0.005	0
Nucleotides[b]		
None	>0.10	
ATP	55.0 ± 3	100
UTP	19.3 ± 2	35
GTP	7.2 ± 0.5	13

The reaction was carried out in 50 mM HEPES, pH 7.4, with 10 μg cytosolic proteins as described in section 2. $PolyGlu_4Tyr$ (0.67 mg/ml) was added subsequent to 30 min preincubation of the enzyme source with ATP (100 μM) and indicated divalent cations (a), or with the indicated nucleotides (100 μM), Mg^{2+} (10 mM), and Co^{2+} (2 mM) (b).

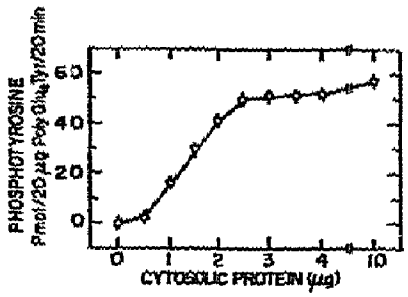

Fig. 1. Phosphorylation of $PolyGlu_4Tyr$ as a function of the concentration of adipocyte crude extract. The crude extract was prepared as described in section 2. The tyrosine kinase assay mixture (60 μl final volume in 50 mM HEPES buffer, pH 7.4) contained 0.1 mM ATP, 15 mM $MgCl_2$, 2 mM Co^{2+}, $PolyGlu_4Tyr$ (0.67 mg/ml) and the indicated concentrations of cytosolic protein. The substrate was added subsequent to a preincubation period of 30 min and the reaction proceeded for 20 min at 22°C. The amount of phosphate incorporated into $PolyGlu_4Tyr$ was estimated by RIA.

3.2. *Quantitation*

Figure 1 represents the extent of $PolyGlu_4Tyr$ phosphorylation at increasing concentrations of high-speed supernatant fraction. No detectable phosphorylation was observed at 0.1–0.2 μg cytosolic protein. Phosphorylation was linearly increased at the range of 0.5 to 2.5 μg cytosolic protein. The specific activity amounted to 20 ± 2 pmol PTyr/20 μg $PolyGlu_4Tyr$/20 min/μg cytosolic protein. This is an extremely high activity; compar-

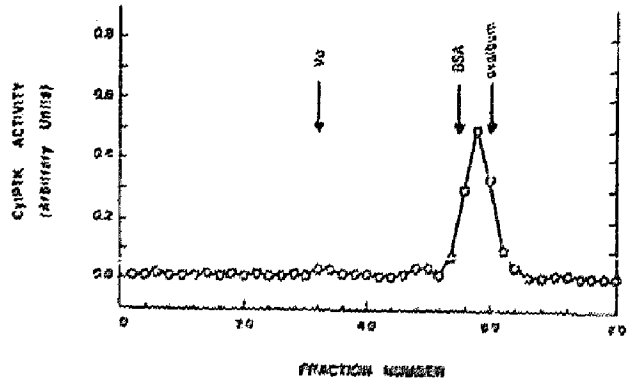

Fig. 2. Elution pattern of CytPTK activity on Sephadex G-200 column. Partially purified CytPTK was loaded on Sephadex G-200 column (1.3 × 60 cm), equilibrated and eluted with 50 mM HEPES, pH 7.4. Aliquots of each fraction (0.5 ml) were assayed for CytPTK activity under standard conditions as described in section 2.

ison of the PolyGlu$_4$ Tyr-phosphorylating activity of the detergent-soluble plasma membrane fraction (i.e. that of IRTK [14,15]) to that of the cytosol seems to indicate that the cytosolic PolyGlu$_4$Tyr-phosphorylating capacity of adipocytes exceeds that of the plasma membrane by more than one order of magnitude (not shown).

3.3. *Partial purification of CytPTK activity*

About 100-fold purification of CytPTK was obtained by two consecutive purification steps. Chromatography on Cibracon blue-agarose (which binds proteins having ATP-binding domains) led to ~20-fold purification and subsequent step of gel filtration resulted in further ~5-fold purification (Table II). The enzyme activity emerged as a single peak from Sephadex G-200 column, suggesting predominantly one species of soluble tyrosine kinase in rat adipocytes with molecular mass in the vicinity of 53 ± 3 kDa (Fig. 2).

3.4. *CytPTK is not related to IRTK*

In addition to the different requirement for divalent ions (Table I), and different molecular mass (Fig. 2), CytPTK differed from IRTK at least in 3 more criteria: (i) the enzyme was not inactivated by *N*-ethylmaleimide (Table III); (ii) it was effectively inhibited by staurosporine (ED_{50} ≈ 3 nM, Table III); and (iii) its specific activity was increased 3–4-fold when high-speed supernatant was obtained from vanadate-pretreated adipocytes (Fig. 3). Neither of these criteria is valid for IRTK (Table III and [13,16,17]) strongly supporting the view that these two tyrosine kinases do not relate at all to each other.

3.5. *CytPTK activation is unique to vanadate ions*

The fact that vanadate, which is a potent inhibitor of tyrosine dephosphorylation [18,19] enhanced largely CytPTK activity (Fig. 3), may reflect the necessity of tyrosine phosphorylation for the maximal expression of the enzyme activity. This conclusion is supported further by the observation that pretreatment of the crude extracts with immobilized alkaline phosphatase

Table II

Partial purification of CytPTK from rat adipocytes

Purification step	Protein mg	Activity units[a]	Specific activity units mg^{-1}	Purification -fold	Yield (%)
40,000 × g supernatant	9.6	8,000	830	1	100
Cibacron blue 3GA-agarose	0.41	7,370	17,970	22	92
Sephadex G-200	0.038	3,100	81,580	98	39

[a] One unit is defined as the amount of the enzyme which produces 20 pmol PTyr per 20 min under standard assay conditions described in section 2.

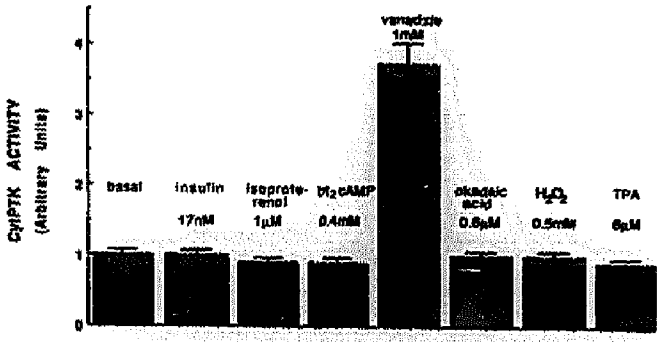

Fig. 3. Effect of insulinomimetic and counter-regulating agents on CytPTK activity. Adipocytes (5 × 10^6 cells/ml) were pretreated with the indicated reagents for 40 min at 37°C, or for 5 min with insulin. The cells were disrupted by freezing/thawing procedure and the crude extracts were collected as described in section 2. The tyrosine kinase activity was evaluated by the amount of incorporated phosphate in PolyGlu$_4$Tyr as described in Fig. 1.

markedly reduced the phosphorylating capacity of CytPTK (Table III). Alkaline phosphatase of animal and bacterial origin has been reported to exhibit high specificity toward phosphotyrosine substrates at neutral pH [20,21]. Whether CytPTK by itself undergoes activation by autophosphorylation as do many of the tyrosine kinases described to date [2,3,7], is a matter of further studies. The large stimulation of CytPTK activity observed in vanadate-pretreated cells seems to be a unique feature of this insulinomimetic agent. The other insulinomimetic agents studied here including okadaic acid [22,23], H_2O_2 [24,25], TPA [26] and insulin by itself, as well as some agents that counterregulate the actions of insulin such as isoproterenol and bt$_2$cAMP [27] failed to alter CytPTK activity when applied to intact adipocytes (Fig. 3). It could be concluded therefore that

Table III

Effects of different treatment on CytPTK and IRTK activity

Conditions	pmol PTyr/20 μg PolyGlu$_4$Tyr/20 min.	Relative activity %
CytPTK activity[a]		
Basal	20 ± 3	100
NEM, 1 mM	32 ± 2	96
Staurosporine, 3 nM	10 ± 2	50
Staurosporine, 10 nM	2.0 ± 0.3	10
Alkaline phosphatase (7 units/ml)[b]	>1 ± 0.7	>5
IRTK (liver)[c]		
Basal	6.4 ± 3	100
NEM, 1 mM	3.5 ± 2	5.4
Staurosporine, 2 μM	61 ± 2	95
Staurosporine, 10 μM	24 ± 1.4	38

[a] Assayed with 1 μg cytosolic protein in the presence of indicated inhibitors, Mg^{2+} (10 mM) and Cl^{2+} (2 mM), as described in section 2. [b] Immobilized alkaline phosphatase was added prior to the assay for 30 min at 37°C and was removed thereafter by centrifugation. [c] Assayed with 1 μg WGA-agarose purified IRTK in the presence of indicated inhibitors, Mg^{2+} (10 mM), Mn^{2+} (2 mM), 10^{-7} M insulin, and 0.1% Triton X-100.

CytPTK activity is not affected under conditions that elevate cellular cAMP levels, increase serine/threonine phosphorylation state of cellular proteins, activate protein kinase C or affect cellular events mediated by insulin.

As mentioned above, the role of nonreceptor PTKs found in other mammalian tissues is still unknown [7]. Our observation that VO^{-}_{3} activates CytPTK supports the view that this enzyme participates in the vanadate-dependent effects in activating carbohydrate and lipid metabolism in rat adipocytes. As shown previously, vanadate mimics virtually all the biological effects of insulin in this cell type, probably in a postreceptor manner as it does not modify insulin receptor function [17]. It is tempting to speculate therefore that the lack of stimulation of CytPTK by insulin may reflect the differences in the mode of action between insulin and vanadate on carbohydrate and fat metabolism. However, further studies will shed light on the participation of the nonreceptor PTK in the metabolism of normal adipocytes.

REFERENCES

[1] Hunter, T. and Sefton, B.M. (1980) Proc. Natl. Acad. Sci. USA 77, 1311–1315.
[2] Yarden, Y. and Ullrich, A. (1988) Annu. Rev. Biochem. 57, 443–478.
[3] Hunter, T. and Cooper, J.A. (1985) Annu. Rev. Biochem. 54, 897–930.
[4] Brunati, A.M. and Pina, L.A. (1988) Eur. J. Biochem. 172, 451–457.
[5] Clari, G., Brunati, A.M. and Moret, V. (1988) Eur. J. Biochem. 175, 673–678.
[6] Khan, N.A., Masson, L., Quermener, V., Clari, G., Moret, V. and Moulinoux, J.P. (1990) Biochem. Int. 20, 863–868.
[7] Srivastava, A. (1990) Int. J. Biochem. 11, 1229–1234.
[8] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[9] Glieman, J., Osterlund, K., Vinten, J. and Gammeltoft, S. (1972) Biochim. Biophys. Acta 286, 1–9.
[10] Bradford, M.M. (1976) Clin. Chem. 20, 1255–1271.
[11] Shisheva, A., Leitner, O. and Shechter, Y. (1991) J. Biochem. Biophys. Methods 23, 307–314.
[12] Meyerovitch, J., Kahn, R.C. and Shechter, Y. (1990) Biochemistry 29, 3654–3660.
[13] Czech, M.P. (1985) Annu. Rev. Physiol. 47, 357–381.
[14] Cuatrecasas, P. (1972) J. Biol. Chem. 247, 1980–1991.
[15] Jacobs, S., Shechter, Y., Bissel, K. and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 77, 981–990.
[16] Ridge, H.K., Hofman, K. and Finn, F.M. (1988) Proc. Natl. Acad. Sci. USA 85, 9489–9493.
[17] Shechter, Y. (1990) Diabetes 39, 1–5.
[18] Nelson, R.L. and Branton, P.E. (1984) Mol. Cell. Biol. 4, 1003–1012.
[19] Swarup, G., Cohen, S. and Garbens, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104–1109.
[20] Swarup, G., Cohen, S. and Garbens, D.L. (1981) J. Biol. Chem. 256, 8197–8201.
[21] Heng-Chun, L.I., Chernoff, J., Chen, L.B. and Kirshonbaum, A. (1984) Eur. J. Biochem. 138, 45–51.
[22] Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Trends Biochem. Sci. 15, 98–102.
[23] Shisheva, A. and Shechter, Y. (1991) Endocrinology 129, 2279–2287.
[24] Czech, M.P., Lawrence Jr., J.C. and Lynn, W.S. (1974) Proc. Natl. Acad. Sci. USA 71, 4173–4177.
[25] Little, S.A. and de Haen, C. (1980) J. Biol. Chem. 255, 10888–10895.
[26] Cherqui, G., Caron, M., Wicek, D., Lascols, O., Capeau, J. and Picard, J. (1986) Endocrinology 118, 1759–769.
[27] Khoo, J.C., Aquino, A.A. and Steinberg, D. (1974) J. Clin. Invest. 53, 1124–1131.