Vol. 116, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 31, 1983 Pages 400-407

# TYROSINE PROTEIN KINASE ACTIVITY IN PURIFIED RAT LEYDIG CELLS

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Received August 30, 1983

Tyrosine protein kinase activity has been estimated in purified testicular cells with the synthetic peptide substrate $NH_2$-GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY-COOH. High levels of enzyme specific activity (56-165 pmol/mg/min) were found in the two populations of Leydig cells isolated by Metrizamide gradient centrifugation. Some activity was also detected in germinal cells, red cells and seminiferous tubules from testis but at levels 6-20 times lower than those found in the Leydig cell fractions. Higher levels of tyrosine protein kinase specific activity were found in population I than in population II Leydig cells.

Tyrosine protein kinases are a unique group of enzymes that specifically phosphorylate tyrosine residues of protein substrates (1,2). These protein kinase activities have been found associated with retrovirus transforming proteins such as $pp60^{src}$ of Rous sarcoma virus (3) as well as a number of mitogenic, polypeptide growth factor receptors including those for epidermal growth factor (2,4), insulin (5-7), platelet-derived growth factor (8-10), and insulin-like growth factor 1 (7). These results suggest a relationship

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[1]Abbreviations used: $E_{11}G_1$, $NH_2$-GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY-COOH; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Hepes, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGF, epidermal growth factor; hCG, human choriogonadotropin; NP-40, Nonidet-P40; 3β-HSD, 3β-hydroxysteroid dehydrogenase; M199-BSA, medium 199, containing 0.1% bovine serum albumin.

0006-291X/83 $1.50

between tyrosine protein kinase activity and the control of cell growth, although the exact role of the enzyme is not known.

It was recently shown that a substantial amount of tyrosine protein kinase activity is present in various tissues of the rat including testes when an exogenous peptide is used as substrate (8). Herein, a synthetic peptide substrate, $NH_2$-GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY-COOH, is utilized to measure tyrosine-specific protein kinase activity in different cellular fractions of the rat testis and, specifically, in two purified populations of Leydig cells.

## EXPERIMENTAL PROCEDURES

Materials. Male Sprague-Dawley rats (70-90 days) were obtained from Sasco (Omaha, NE). Metrizamide (centrifugation grade, Nyegaard, Norway) was supplied by Accurate Chemical and Scientific Corp. (Hicksville, NY). Collagenase type I (131 u/mg) and Medium 199 were purchased from Millipore (Worthington) Corp. (Freehold, NJ) and Grand Island Biological Supply (Grand Island, NY), respectively. [$\gamma$-$^{32}$P]ATP was prepared by a published procedure (9). All peptide substrates used were synthesized by solid phase methods using the Merrifield resin (10) as previously described by Garbers et al. (11) and purified by high performance liquid chromatography. All other chemicals were of the highest reagent grade and obtained from standard suppliers. Mouse EGF was a generous gift of Dr. Stanley Cohen, Vanderbilt University. Purified rat prolactin (rPrl B-2) and hCG (preparation CR-121) were supplied under the auspices of the National Hormone and Pituitary Program.

Isolation of Leydig Cells and Preparation of Tyrosine Protein Kinase-Containing Particles. Sixteen rats were sacrificed by decapitation for each preparation. Leydig cells, germinal cells and red cells were isolated on discontinuous Metrizamide gradients by the method of O'Shaughnessy et al. (12). One ml fractions were collected from the top of each gradient, pooled and washed free of Metrizamide with M199-BSA[1]. Individual tubules were collected after collagenase treatment and washed 2X with M199-BSA. Staining for 3$\beta$-HSD was performed according to Wiebe (13). The cells which contained dark blue formaxan deposits, indicating the presence of 3$\beta$-HSD, were considered Leydig cells. Cells, isolated tubules and whole testes were homogenized in an ice cold solution containing 10 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.2, with a Polytron (Brinkman) at setting 5 for 3 x 10 sec intervals. The homogenates were centrifuged at 1000xg for 6 min (4°C) to remove nuclei and unbroken cells. The resulting supernatant fluids were centrifuged at 30,000xg for 25 min at 4°C to prepare soluble and particulate fractions. The pellets containing most of the tyrosine protein kinase activity were resuspended in the Hepes buffer by homogenization as described above or with 10 strokes of a teflon/glass tissue homogenizer and stored frozen at -35°C until assayed. Protein was determined according to Bradford (14) using gamma globulin as a standard.

Peptide Phosphorylation Assay. Phosphorylation of synthetic peptides was performed as described by Swarup et al. (8). The reaction mixture contained 50 mM Tris-HCl, pH 7.8, 50 mM $Mg^{2+}$, 10 $\mu$M vanadate, 60 $\mu$M [$\gamma$-$^{32}$P]ATP (~1500 cpm/pmol) and 1 mM $E_{11}G_1$ or another peptide. In experiments to determine the effect of hormones on tyrosine protein kinase activity, the particulate fractions from Leydig I and II cells were pre-incubated with $10^{-8}$ M hormone at 24°C for 1 h (prolactin and hCG) or 30 min at 0-4°C (EGF)

in Hepes buffered saline, pH 7.2, containing 1 mM $MgCl_2$ to allow binding of the hormones to membrane-associated receptors (15,16). Aliquots of the pre-incubation mixtures were then assayed as described above in the presence or absence of NP-40.

Endogenous Phosphorylation and SDS-PAGE Electrophoresis. The phosphorylation of endogenous proteins was performed at 30°C in 100 µl of 50 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 10 µM vanadate, 10 µM [$\gamma$-$^{32}$P]ATP (30-50,000 cpm/pmol), and an aliquot of particulate material (35-150 µg) in the absence or presence of 0.1% NP-40. The reaction was terminated after 5 min by the addition of 100 µl of sample incubation buffer containing 6 % SDS, 20 % glycerol, 10 % β-mercaptoethanol and 125 mM Tris-HCl at pH 6.8. Samples were incubated at 100°C for 3 min and analyzed by SDS-PAGE in 1.5 mm slabs with the discontinuous buffer system described by Laemmli (17). Gels were treated with 1 M KOH at 56°C for 2 h as previously described (18). Radioactivity was visualized by autoradiography using Kodak X-Omat R film.

## RESULTS

Tyrosine Specific Protein Kinase Activity in Purified Leydig Cells.

Testicular cells were separated by the discontinuous Metrizamide gradients into several bands based upon bouyant density (15) which, listed from the top of the gradient, were designated germinal cells, Leydig I cells, Leydig II cells and red blood cells. The percentage of Leydig cells in these four fractions as determined by staining for 3β-HSD were 1.5 ± 1.5, 24.5 + 2.9, 91.9 ± 4.0 and 3.9 ± 1.2, (mean ± S.D.; n = 3) respectively. The tyrosine specific protein kinase activity in the particulate fractions from the four cell types, as well as whole testes and seminiferous tubules, was estimated using the synthetic peptide substrate $E_{11}G_1$. As represented in Fig. 1 the highest tyrosine protein kinase specific activity was detected in Leydig cell populations I and II. Measurable tyrosine protein kinase activity was present in the other particulate fractions tested, although at levels 6-20 times lower than the levels found in the Leydig cells. Greater than 90 % of the measured enzyme activity was associated with the 30,000xg pellets (Table I). Protein concentration curves for the particulate fractions from Leydig cell populations I and II are similar and linear to at least 8-10 µg protein/assay tube (Fig. 2A). The amount of phosphorylated peptide formed was linear with time for at least 15 min (Fig. 2B).

Effect of Substrate on Tyrosine Protein Kinase Activity. The effect of amino acid deletions or insertions in the peptide substrates on specific activity was measured in the particulate fractions from the Leydig cell

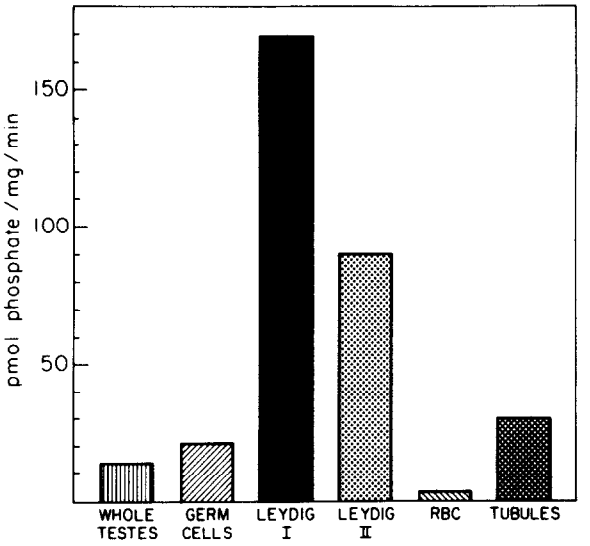


Figure 1: Tyrosine protein kinase activity in particulate fractions prepared from purified cells from normal rat testes (see Experimental Procedures). All assays performed at 30°C for 10 min with 60 μM [$\gamma$-$^{32}$P]ATP, 50 mM $MgCl_2$, 10 μM orthovanadate, 1 mM $E_{11}G_1$ (peptide) and 0.02 % NP-40.

populations and is presented in Table II. A comparison of activities using $E_{11}G_1$ and $E_{10}G_1$ (see Table II for structure) suggest that an additional arginine residue doubles the apparent specific activity of the

TABLE I

TYROSINE SPECIFIC PROTEIN KINASE ACTIVITY IN FRACTIONS FROM LEYDIG CELL POPULATIONS I AND II OF RAT TESTES[1]

| | Fraction | Specific Activity[2] (pmol/mg/min) | |
|---|---|---|---|
| Leydig I | 1000xg pellet | 8.1 | (7.1-9.1) |
| | 30,000xg supernatant fluid | 7.8 | (5.8-9.8) |
| | 30,000xg pellet | 113 | (56-165) |
| Leydig II | 1000xg pellet | 3.2 | (0.8-5.6) |
| | 30,000xg supernatant fluid | 4.5 | (0-4.5) |
| | 30,000xg pellet | 82 | (31-125) |

[1]Assay performed with 60 μM ATP, 50 mM $MgCl_2$, 10 μM vanadate, and 0.02 % NP-40. Reaction mixtures were incubated at 30°C for 10 min.

[2]Mean of 2 or 3 experiments in each case. The range is indicated in parentheses.

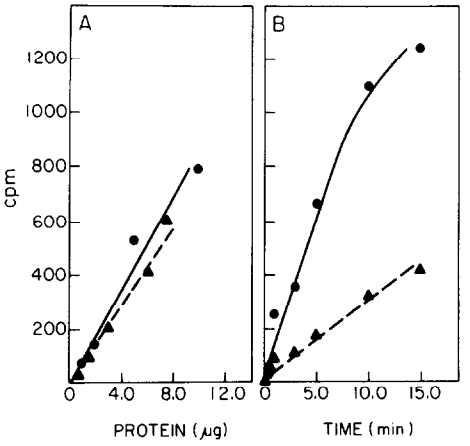


Figure 2: (A) Effect of protein concentration on tyrosine protein kinase activity in particulate fractions from normal rat Leydig cells. All assay conditions as described in legend to Figure 1. o-o, Leydig population I; Δ-Δ, Leydig population II. (B) Time course of peptide phosphorylation by particulate fractions from normal rat Leydig cells. Assays performed with 1.9 and 1.5 μg of protein from Leydig I and Leydig II particulate fractions, respectively.

enzyme. There is little change in activity with the addition of a single isoleucine residue at the $NH_2$-terminus. As expected, replacement of the single tyrosine with phenylalanine ($E_{10}$-F-$G_1$) eliminates phosphorylation of the peptide.

TABLE II
EFFECT OF PEPTIDE STRUCTURE ON TYROSINE KINASE PROTEIN ACTIVITY IN RAT LEYDIG CELL PARTICULATE FRACTIONS

| | Structure | Specific Activity (pmol/mg/min) | |
|---|---|---|---|
| | | Leydig I | Leydig II |
| A8-G1 | ALA-GLU-TYR-ALA-ALA-ARG-ARG-GLY | 24 | 24 |
| E10-G1 | GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-GLY | 32 | 50 |
| E10-F-G1 | GLU-ASP-ALA-GLU-PHE-ALA-ALA-ARG-ARG-GLY | 0 | 0 |
| E11-G1 | GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY | 57 | 104 |
| I12-G1 | ILE-GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY | 49 | 92 |
| L13-G1 | LEU-ILE-GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY | 25 | 18 |

All assays performed at a peptide concentration of 1 mM with 60 μM [$\gamma$-$^{32}$P]ATP, 50 mM $MgCl_2$, 10 μM vanadate and 0.02 % NP-40. The amount of protein assayed was 2.0 μg and 1.5 μg for population I and II Leydig cell particulate fractions, respectively. The amino terminal residue is shown to the far left in each peptide.

The phosphorylation of $E_{11}G_1$ was not stimulated by the addition of 10 nM mouse EGF, rat prolactin or hCG under the conditions tested.

Phosphorylation of Endogenous Proteins. Particulate material from the Leydig I cell population incubated in the presence of [$\gamma$-$^{32}$P]ATP and analyzed by SDS gel electrophoresis indicated that many protein bands were phosphorylated. Two prominent bands were detected with apparent molecular weights of 49,000 and 54,000 (Fig. 3A). Several other minor radiolabeled bands at 73,000, 79,000, 101,000, 122,000 and 133,000 were also present. A major radiolabeled band was detected with an apparent molecular weight of 52,500. Autoradiographs of gels which had been previously treated with alkali typically displayed one major radiolabeled band of approximately 48,000 molecular weight and several minor radiolabeled bands with higher apparent molecular weights (Fig 3B).

## DISCUSSION

Utilizing the synthetic peptide substrate $NH_2$-GLU-ASP-ALA-GLU-TYR-ALA-ALA-ARG-ARG-ARG-GLY-COOH we have demonstrated the presence of high tyrosine

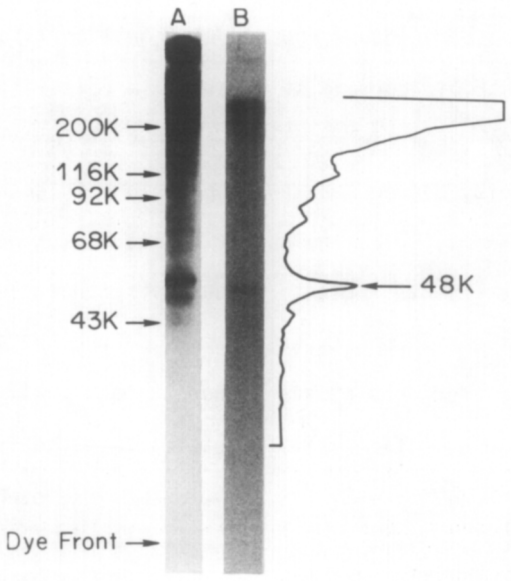


Figure 3: Autoradiograph of a 10% SDS slab gel of phosphorylated endogenous proteins in the particulate fraction from Leydig I cells. Samples were incubated in the presence of 20 $\mu$M [$\gamma$-$^{32}$P]ATP as outlined in Experimental Procedures. Lane a represents a non-alkali treated gel in the presence of 0.1% NP-40; Lane b represents an identical gel treated with alkali as described in Experimental Procedures. The inset shows a densitometric scan (LKB Laser Scanning Densitometer) of the autoradiograph.

protein kinase activity (56-165 pmol/mg/min) in purified Leydig cells from rat testis relative to other testicular cells. Activity was also detected in the germinal cells, red cells and seminiferous tubules from testis but at levels 6-20 times lower than those found in the Leydig cells. Previously, Swarup et al. (8) demonstrated tyrosine protein kinase (14 pmol/mg/min) activity in whole rat testis utilizing a related synthetic peptide substrate. Peptide substrates similar to the phosphorylation sequence of $pp60^{src}$ also have been used by others to study the EGF receptor kinase (19), insulin-stimulated tyrosine phosphorylation (5), viral oncogene products (20) and tyrosine protein kinase activity during embryogenesis (21). Peptides of different structure also have been used to estimate tyrosine protein kinase activity (22).

These findings are of interest in that the Leydig cell is the principal androgen biosynthetic cell of the testis. Recent evidence indicates that Leydig cells exist as two putative populations (designated I and II) (12,23,24,25). In sexually mature rats, both populations of cells have the same number of lutropin receptors, but population I cells are much less responsive to gonadotropic hormone stimulation in relation to testosterone production (12,24,26). Our findings also suggest differences in tyrosine protein kinase activity between the two populations in that population I routinely exhibits nearly twice the specific activity of population II. Whether or not these results indicate actual differences in enzyme activity in the two cell populations is not known, however, since the percentage Leydig cells in the two isolated populations is not the same. If the data reflect actual differences in specific activity between Leydig cell populations I and II, possibly the altered enzyme activity is due to the stage of cell maturation. From the results of a developmental study, Chase and Payne suggested that population II Leydig cells are derived from population I Leydig cells during sexual maturation (24,25). Dasgupta et al. (21) have reported increased tyrosine specific protein kinase activity in the developing sea urchin embryo and tyrosine protein kinase specific activity appears to be much

higher in spleen than in thymus (8). Thymus contains mostly immature lymphocytes whereas spleen contains a more mature population.

Although tyrosine protein kinase activity has been suggested as being important in the processes of cell transformation and cell growth (2,27), its function remains unclear. Further studies on Leydig cells and other normal cells may help elucidate the physiological role of tyrosine protein kinases.

ACKNOWLEDGMENTS

We thank D. Janette Tubb and Rae Ann Bellet for their expert technical assistance with the tyrosine protein kinase assays. We also thank Drs. Ghanshyam Swarup and Jai Dev Dasgupta for helpful discussions as well as Diane Smithson for expert secretarial skills. This work was supported in part by grants from the National Institutes of Health (Research Grant AM 15838 and Training Grant CA 090313 to M.H.M.).

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