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Polylysine activates and alters the divalent cation requirements of the insulin receptor protein tyrosine kinase

Ora M. Rosen[+] and David E. Lebwohl[+*]

[+] *Program in Molecular Biology* and [*] *Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue/Box 97, New York, NY 10021, USA*

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Protamine and poly(Lys) activate the protein tyrosine kinase of both the human placental insulin receptor and its purified recombinant cytoplasmic domain. Spermidine, poly(Arg) (average molecular mass 15 kDa), poly(Glu), Arg or Lys are not effective. Activation is stable, reversible, and optimal when the enzyme is preincubated with activator, divalent cation and ATP prior to the addition of exogenous protein substrates. The most striking feature of the activation is that it results in 20–30-fold stimulation of the kinase in the presence of 0.2–0.4 mM Mn^{2+} and induces equivalent activity in the presence of Mg^{2+} alone (0.4–4.0 mM). The activated protein tyrosine kinase has a specific activity (0.25–0.5 μmol/mg protein) that approaches that of well characterized protein serine kinases.

Insulin receptor; Protein tyrosine kinase; Polylysine

1. INTRODUCTION

We have previously reported the overproduction and purification of the insulin receptor's cytoplasmic domain in a Baculovirus expression system [1]. The latter protein, BIRK, has an M_r of 48000, autophosphorylates and is as active on exogenous substrates as the native insulin receptor. Two features of both the insulin receptor and BIRK, shared by a number of other protein tyrosine kinases, are (i) relatively low specific activity (BIRK catalyzes the incorporation of 20 nmol ^{32}P from [γ-^{32}P]ATP into histone/min per mg protein) and (ii) dependency upon Mn^{2+} as the obligatory divalent cation. We now report that either protamine or poly(Lys) dramatically stimulate the protein tyrosine kinase activity of both the insulin receptor and BIRK rendering it active in the presence of either Mg^{2+} alone or more physiological concentrations of Mn^{2+}.

Correspondence address: O.M. Rosen, Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue/Box 97, New York, NY 10021, USA

2. MATERIALS AND METHODS

Poly(Lys) (average molecular masses 17, 14, 4.4 and 3.5 kDa), poly(Arg) (average molecular masses 43 and 15 kDa), poly(Glu) (molecular mass 17 kDa), Lys, Arg, protein A-sepharose were from Sigma. Protamine was from Eli Lilly Inc. Histone H2b, casein, spermidine were from Boehringer Mannheim. The preparation of MAPS plus tubulin was provided by Dr R.B. Vallee [2] and brain fodrin by Dr Paul Wagner (see [3]). [γ-^{32}P]ATP (3000 Ci/mmol) was from New England Nuclear Corp. Antipeptide antibodies are detailed in [4] and the monoclonal antibody in [5]. Sources of other materials are given in [1]. Unless otherwise indicated, the preparations of poly(Lys) had average molecular masses of 14 kDa.

At least five independent preparations of BIRK were used. The human insulin receptor was purified on wheat germ agglutinin-agarose [6].

2.1. *Protein kinase assays*

BIRK and the insulin receptor were assayed at 30°C in 25–30 μl [1]. Quantitation was achieved by cutting out phosphoproteins after PAGE and counting them directly. Immunoprecipitation was performed as in [4]. Alkali treatment of polyacrylamide gels is outlined in [7] and phosphoamino acid analysis in [6].

3. RESULTS AND DISCUSSION

3.1. *Activation of BIRK*

As shown in fig.1, protamine induces the

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autophosphorylation of BIRK in the presence of 8 mM Mg^{2+}; 1–2 mol P were incorporated per mol BIRK. In the presence of either Mg^{2+} (2.7 mM) or Mn^{2+} (0.4 mM) substrate phosphorylation is enhanced. In fig.2, five receptor substrates were evaluated in the presence or absence of 47 nM poly(Lys). In addition to the activation, kinase activity is similar with either Mg^{2+} or Mn^{2+}. All substrates were phosphorylated on tyrosyl residues only (not shown). There was no activity in the presence of heated BIRK or in the absence of either BIRK or divalent cations. The specific activity of the activated enzyme using histone H2b was estimated to be 0.25 μmol and 0.5 μmol/min per mg enzyme in the presence of Mg^{2+} or Mn^{2+}, respectively.

Protamine and poly(Lys) were effective at concentrations of 0.4–2.0 μM and 20–500 nM, respectively. Higher concentrations of both activators inhibited the kinase reaction. Poly(Arg) (average molecular mass 43 and 17 kDa), poly(Lys) with average molecular masses below 5 kDa, poly(Glu) (molecular mass 17 kDa), spermidine, Lys and Arg were ineffective at concentrations between 50 nM and 0.5 mM.

At 40–50 nM poly(Lys), a concentration found to be optimal under most conditions, the concentrations of Mg^{2+} or Mn^{2+} required for maximal stimulation of both autophosphorylation and substrate phosphorylation were 0.4–4.0 mM and 0.2–0.4 mM, respectively (fig.3).

Protein kinase activation by poly(Lys) was most

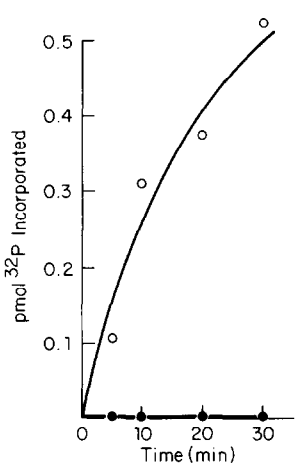


Fig.1. Autophosphorylation of BIRK in the presence of Mg^{2+} and protamine. BIRK (25 ng) was permitted to autophosphorylate in the presence of 8 mM Mg^{2+} plus (—○—) or minus (—●—) 500 ng protamine.

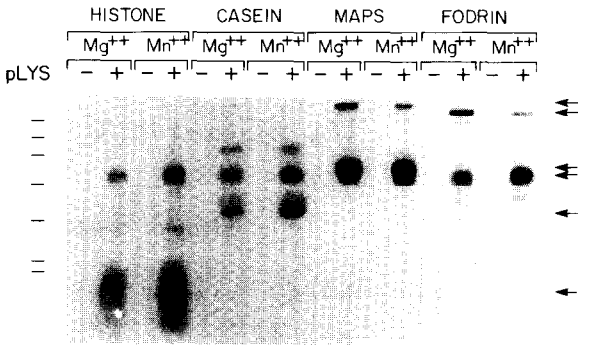


Fig.2. Phosphorylation of exogenous substrates by BIRK in the presence or absence of poly(Lys). BIRK (20 ng) was preincubated with either 2.7 mM Mg^{2+} or 0.4 mM Mn^{2+} in a reaction mixture containing [γ-^{32}P]ATP (35 μM, 10 cpm/fmol) with (+) or without (–) 20 ng poly(Lys) (pLYS) for 15 min at 30°C. Substrates were then added and the kinase reactions allowed to proceed for 5 min at 30°C. Products were identified following PAGE and radioautography for 1 h at –70°C. The content of substrates per assay were 25 μg histone H2b, 25 μg casein, 5 μg MAPS plus tubulin and 1 μg fodrin. The positions of the molecular mass markers (205, 97, 68, 45, 29, 14 and 8 kDa, respectively) are on the left. Arrows on the right point (from top to bottom) to phosphorylated MAPS, fodrin, tubulin, BIRK, casein and histone.

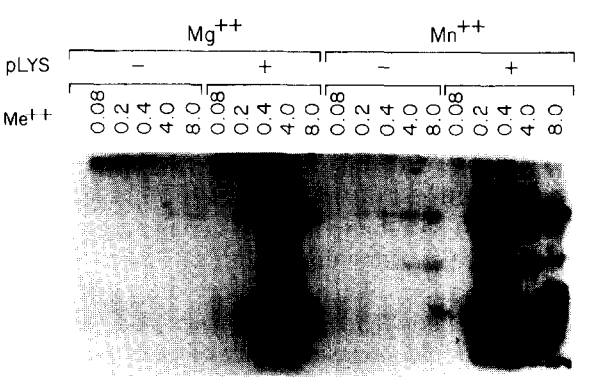


Fig.3. Effect of divalent cation concentration on activation of BIRK by poly(Lys). BIRK (20 ng) was incubated at the indicated concentrations of divalent cations (Me^{2+}) in the presence or absence of 20 ng poly(Lys) and the standard components of the protein kinase reaction mixture. After 15 min at 30°C, 10 μg of histone were added to each tube and the incubations were continued for 7 min. Following PAGE, radioautography was for 60 min at –70°C. The principal phosphoproteins visible are autophosphorylated BIRK (48 kDa) and histone.

striking when the enzyme was preincubated with the polymer, ATP and divalent cation for 5–15 min at 15–37°C prior to introduction of exogenous substrate (fig.4).

BIRK was activatable in washed immunoprecipitates (see fig.5). Antibodies to either the C-terminus (AbP5) or N-terminus (AbP4) (not shown) of the protein gave the same results as did experiments with Mg^{2+} instead of Mn^{2+}. These data are consistent with a model in which poly(Lys) interacts directly with a single or at least constrained molecules of BIRK. This also makes it unlikely that poly(Lys) removes an inhibitor or protein phosphatase contaminant in the enzyme. When activated BIRK is diluted prior to the addition of substrate, it loses activity but can be completely reactivated by the addition of poly(Lys) (not shown).

With tubulin or histone as substrates, 0.4 mM Mn^{2+} and 47 nM poly(Lys), the concentrations of ATP required for half-maximal activation were 30 and 10 μM, respectively, whereas in the absence of poly(Lys), the concentration of ATP required for histone phosphorylation was 50 μM. With 0.4 mM Mg^{2+} and poly(Lys) the concentration of ATP required for half-maximal phosphorylation of tubulin was 100 μM.

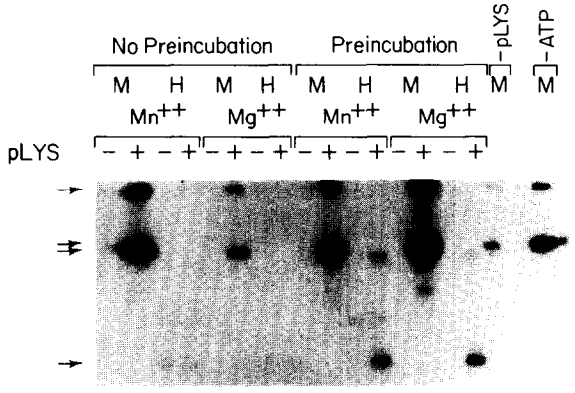


Fig.4. Effect of preincubation on activation of BIRK by poly(Lys). BIRK (10 ng) was assayed with either Mg^{2+} (8.3 mM) or Mn^{2+} (4 mM) with (+) or without (−) 500 ng poly(Lys). In the first 8 lanes, all components including either 5 μg MAPS plus tubulin (M) or 25 μg histone (H) were incubated for 15 min at 30°C. In the next 8 lanes, the components of the reaction mixture were incubated for 15 min at 30°C following which substrates were added and the incubation continued for 5 min at 30°C. In the last 4 lanes, preincubations were performed either in the absence of poly(Lys) (lanes 17 and 18) or in the absence of ATP (lanes 19 and 20). For these last 4 lanes, only MAPS plus tubulin were used as substrates and alternate lanes were preincubated with either Mn^{2+} or Mg^{2+}. The components of the reaction mixture were completed at 15 min, prior to the final 5 min assay. The arrows point (from top to bottom) to phosphorylated MAPS, tubulin, BIRK and histone.

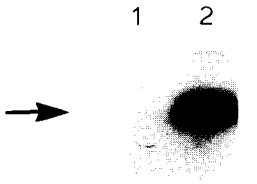


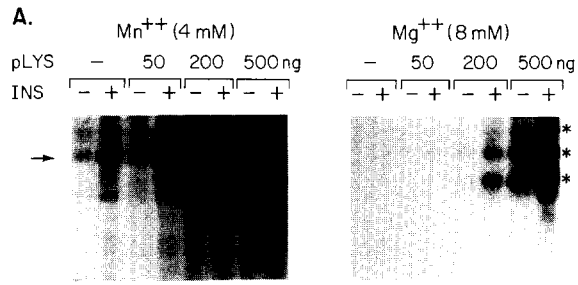
Fig.5. Effect of poly(Lys) on the autophosphorylation of immunoprecipitated BIRK. Purified BIRK was immunoprecipitated with AbP5 and the washed immunoprecipitates were assayed with 1.5 mM Mn^{2+} in the absence (lane 1) or presence (lane 2) of poly(Lys) (2 μg/ml) for 15 min at 30°C. The reaction was analyzed by SDS-PAGE. Similar results were obtained with 0.2 mM Mn^{2+} or 3 mM Mg^{2+}.

3.2. *Effect of poly(Lys) on the activity of the human placental insulin receptor*

When poly(Lys) was added to partially purified insulin receptor in the presence of either 4 mM Mn^{2+} or 8 mM Mg^{2+}, insulin-dependent, alkali stable phosphorylation was enhanced (fig.6A). One of the radioactive bands (arrow) was immunoprecipitable by an antibody to the receptor (fig.6B) and was phosphorylated predominantly on tyrosyl residues (fig.6C). The other proteins phosphorylated in response to insulin were also phosphorylated on tyrosyl residues (not shown). Finally (fig.7), the poly(Lys)-activated insulin receptor was able to phosphorylate exogenous substrates in the presence of Mg^{2+}.

Polyamines, protamine and/or poly(Lys) have previously been reported to activate soluble (see [8–10]) and membrane-associated protein kinases [11,12]. Certain protein phosphatases (see [13]) may also be activated.

The results presented here indicate that poly(Lys) or protamine activate the protein tyrosine kinase activity of both the insulin receptor and BIRK. Activation is apparent with different preparations of enzyme, protamine and poly(Lys) and at all concentrations of ATP (up to 2.0 mM). The most profound effect is activation of the in-



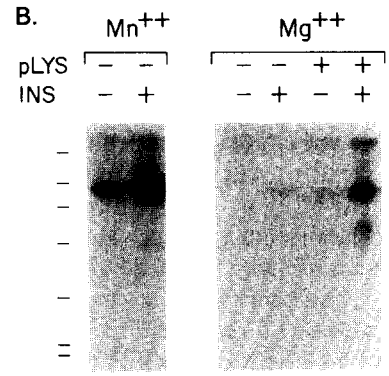
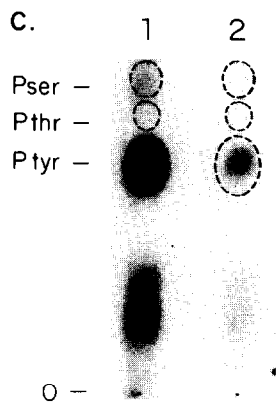


Fig.6. Effect of poly(Lys) on insulin-dependent phosphorylation. (A) Human placental wheat germ agglutinin eluate (3 μg) was incubated (+) or (−) 100 ng insulin (INS) for 15 min at 23°C. Poly(Lys) was then added for 15 min at 30°C. The arrow points to the β-subunit of the receptor. Following reduction and SDS-PAGE, the gel was treated with alkali prior to radioautography for 60 min at −70°C. The asterisks indicate phosphoproteins whose analyses revealed phosphotyrosine only. (B) Following activation by poly(Lys), reaction products were immunoprecipitated using a monoclonal antibody. After washing, pellets were subjected to SDS-PAGE. The radioautogram was developed for 3 h at −70°C. The lines on the left indicate molecular mass markers (205, 97, 68, 45, 29, 14 and 8 kDa). (C) The β-subunit of the insulin receptor was excised from the gel shown in (B) digested with TPCK-treated trypsin (200 μg/ml), and hydrolyzed in 6 N HCl for 60 min at 100°C. O-, origin; Ptyr-, phosphotyrosine; Pthr-, phosphothreonine; Pser-, phosphoserine. Lanes: 1, plus insulin and Mn^{2+}; 2, plus insulin, poly(Lys), Mg^{2+}. There was no autophosphorylation in the presence of Mg^{2+} without poly(Lys).



sulin receptor's protein kinase activity in the presence of Mg^{2+}. The addition of 30–50 nM poly(Lys) allows the enzyme to function at Mg^{2+} concentrations between 0.4 and 4 mM (fig.3) whereas in its absence, there is essentially no activity.

One explanation for these results is that poly(Lys) (or protamine) is substituting for another molecule that regulates the activity of the insulin receptor's tyrosine kinase. Another non-exclusive possibility is that there are minimally two sites that regulate catalytic activity. One site can be productively occupied by either Mn^{2+} or poly(Lys). With poly(Lys), either Mg^{2+} or Mn^{2+} (0.2–0.4 mM) can combine with ATP to form the Me^{2+}-ATP complex that interacts at the active site in the kinase domain. Poly(Lys) can then substitute for the higher concentrations of Mn^{2+} that appear to be necessary for activation at a second regulatory site.

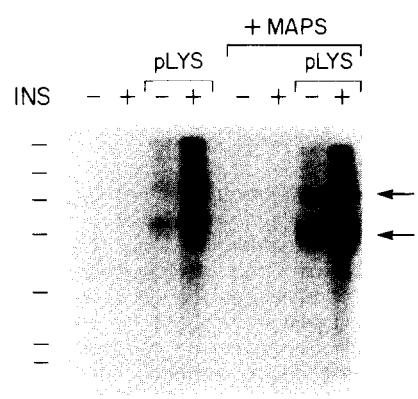


Fig.7. Stimulation of tubulin phosphorylation by the human placental insulin receptor. Assays, in 8 mM Mg^{2+} were performed as described in the legend to fig.6. The last four lanes (+ MAPS) received 5 μg MAPS plus tubulin and were terminated after 10 min. Following PAGE, the gel was subjected to radioautography for 30 min at −70°C. The upper arrow points to the β-subunit and the lower arrow, to phosphorylated tubulin.

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