Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 356-364

PHOSPHORYLATION OF TYROSINE RESIDUES OF RNA POLYMERASE II AND OTHER NUCLEAR PROTEINS BY ACTIVE CHROMATIN TYROSINE KINASE(S)

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Received March 2, 1995

We demonstrate here for the first time that protein tyrosine kinases are present in the active chromatin of nucleus. The presence of tyrosine kinase activity in the active chromatin was initially determined using poly (Glu,Na-Tyr;4:1) (PGT) as a tyrosine phosphorylatable substrate. Active chromatin in the presence of cofactors phosphorylated PGT at a rate of 40 pmol/min. The phosphorylation of PGT by active chromatin was inhibited by 41, 47 and ~95% with genistein, n-ethylmaleimide and quercetin (known inhibitors of tyrosine kinases), respectively. A Lineweaver-Burk plot revealed an apparent Km of 50 μg/ml and Vmax of 45 pmol/min for active chromatin tyrosine kinase(s). Analyses of phosphorylation of endogenous substrates by immunoprecipitation, western blotting and phosphoamino acids revealed that active chromatin protein tyrosine kinase(s) are able to phosphorylate tyrosine residues of the large subunit of RNA pol II and several other active chromatin proteins. The ability of AC-PTKs to phosphorylate many proteins of active chromatin components argues strongly for its role(s) in regulating transcription.

Protein tyrosine kinases catalyze the phosphorylation of tyrosine residues of proteins (1). Most of the tyrosine kinases studied so far have been shown to be associated with growth factors or products of proto-oncogenes, which are mainly located in plasma membrane or cytosol (1). The presence of the activity of tyrosine kinases in the nucleus has not been extensively characterized using exogenous tyrosine phosphorylatable substrate and/or known inhibitors of tyrosine kinases. Based on few

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0006-291X/95 $5.00

known nuclear tyrosine phosphorylated proteins (2), the presence of tyrosine kinases in the nucleus has been suspected.

The presence of several protein kinases such as the protein kinase C, the protein kinase A and the rsk- and erk-encoded protein kinases has been observed in the nucleus (3). The two tyrosine kinases, FER and c-abl have been shown to be localized in both cytosolic and nuclear fractions (4,5). Since these protein kinases (Tyr or Ser/Thr) are also present in the cytoplasm, it is considered that cytoplasmic Tyr or Ser/Thr kinases are translocated into the nucleus in response to stimuli (6). In addition to tyrosine kinases, there are several other nuclear proteins which are phosphorylated on tyrosine residues. Auricchio and colleagues had previously demonstrated that estrogen receptor from calf, rat and human , a nuclear protein, is phosphorylated on tyrosine residues (7). The nuclear androgen receptor protein has also been reported to contain phosphotyrosine (7). Recently, it has been reported that the tyrosine residues of carboxyl terminal domain (CTD) of RNA polymerase II (RNA pol II), a nuclear protein, could be phosphorylated by a tyrosine kinase encoded by the protooncogene c-abl (8). For the tyrosine phosphorylation of proteins residing in the nucleus, these proteins either have to be sequestered into the cytoplasm or tyrosine kinase(s) from the cytoplasm have to be transported into the nucleus. For efficient phosphorylation to occur, it is logical to think that the tyrosine kinases responsible for the phosphorylation of proteins residing in the nucleus should be present in close proximity to their substrates. We now know that there are several tyrosine phosphorylated proteins which reside in the chromatin of the nucleus and are involved in various important nuclear processes (3-8). Therefore, we examined the presence of tyrosine kinase(s) in the active chromatin and whether they can phosphorylate endogenous nuclear proteins such as RNA pol II and other active chromatin proteins at tyrosine residues.

In the present study, we show evidence that protein tyrosine kinases are present in the active chromatin of nucleus. We have supported our results using both exogenous and endogenous substrates for active chromatin protein tyrosine kinases (AC-PTKs). AC-PTKs are able to phosphorylate tyrosine residues of the large subunit of RNA pol II and several other active chromatin proteins.

MATERIALS AND METHODS

Chemicals: Antiphosphotyrosine antibody was from Upstate Biotech. Inc., NY. RNA polymerase II antibody was from Promega Corp., Madison, WI. Aprotinin, genistein,

leupeptin, n-ethylmaleimide, phenylmethyl sulfonyl fluoride (PMSF) and poly (glutamate, Na-tyrosine; 4:1) (PGT) were from Sigma Chemical Co., St. Louis, MO. γ-^{32}P-ATP (4500 Ci/mmol) was from ICN, Costa Mesa, CA.

Preparation of nuclei and active chromatin: Nuclei were prepared from kidneys of 6-8 weeks old male Syrian hamsters (Charles River) by the method of Yu (9). The preparation of nuclei is critical for this study and therefore the purity of the nuclear preparations was assessed both biochemically and morphologically. The activity of glucose 6-phosphatase in the microsomes (positive control) was about 650 pmol/mg protein, which is similar to the previous report (10). The activity of lactate dehydrogenase was less than 0.2% of that found in cytosol (0.02 μmol/mg protein/min in nuclei vs 12.50 μmol/mg protein/min in hamster kidney cytosol). The electron microscopic examination (11) of nuclei suggested that the nuclei were highly pure and cellular contamination was not visible (Fig. 1). All values are in good agreement with the previous observations reported by us (12-14), and suggest that nuclei were highly pure. Active chromatin was prepared by digestion with micrococcal nuclease (100 U/mg DNA) for 10 min (15). The identity of active chromatin in the supernatant was confirmed by the presence of RNA polymerase II by western blotting (Fig. 2).

Active chromatin protein tyrosine kinase(s) were partially purified by immunoprecipitation using phosphotyrosine polyclonal antibody (16). Protein tyrosine kinase activity was assayed using PGT as a phosphorytable substrate (17). Endogenous active chromatin proteins were fractionated on 8% SDS-PAGE by the method of Laemmli (18). The gels were treated with 1 M KOH at 55°C for 1 hr, neutralized and dried under vacuum (19). Phosphoproteins were detected by autoradiography using Kodak X-Omat X-ray film. Phosphotyrosine containing proteins were also detected by western blotting using antiphosphotyrosine polyclonal antibody (20). Phosphoamino acids were separated by 2-dimensional paper chromatography (21). Labelled amino acids were detected by autoradiography.

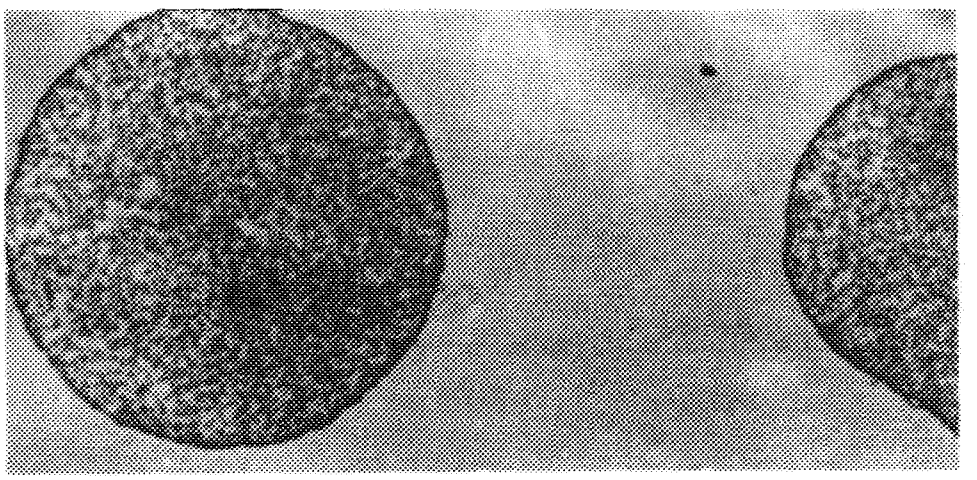

Figure 1. An electron micrograph of purified nuclei (x21,000). The nuclei were isolated (9) and prepared for electron microscopy as described previously (11).

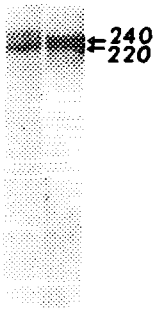

Figure 2. Detection of RNA polymerase II in the active chromatin.
Active chromatin protein (100 μg) was separated on SDS-PAGE. The proteins were transferred to PVDF membrane and the membrane was probed with anti-RNA polymerase II antibody directed against the CTD. Lane 1 = purified calf thymus RNA polymerase IIa; Lane 2 = active chromatin. The molecular weight of the phosphorylated (240 kDa) and the non-phosphorylated (220 kDa) forms of RNA polymerase II are indicated by arrows.

RESULTS

Active chromatin tyrosine kinase(s): Active chromatin in the presence of cofactors phosphorylated PGT at a rate of 40 pmol/min (81,000-84,000 dpm/reaction). In the absence of enzyme or with heat denatured active chromatin, only 1- 2 % of the total radioactive incorporation (800-1500 dpm/reaction) was observed. The phosphorylation of PGT by active chromatin was inhibited by 41, 47, ~95 and 88% with genistein, n-ethylmaleimide, quercetin (known inhibitors of tyrosine kinases) and luteolin, respectively (Table 1). The phosphorylation of PGT by active chromatin was dependent on protein and substrate concentrations, and

TABLE 1
Protein tyrosine kinase activity in the active chromatin

	pmol ^{32}P incorporated/mg Protein	% Inhibition
Active chromatin	1190 ± 76	
+ Genistein (500 μM)	700 ± 82	41
+ N-ethylmaleimide (500 μM)	631 ±	47
+ Luteolin (100 μM)	147 ± 14	88
+ Quercetin (500 μM)	24 ± 4	98

Protein tyrosine kinase activity in the active chromatin was measured by estimating the phosphorylation of PGT (100 μg/ml) using 10 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM sodium-o-vanadate and 25 μg protein/assay in the presence or absence of PTK inhibitors (genistein, N-ethylmaleimide or quercetin) in a final volume of 100 μl buffer, pH 7.5 (17). Each value is a mean of 3-4 measurements ± standard deviation.

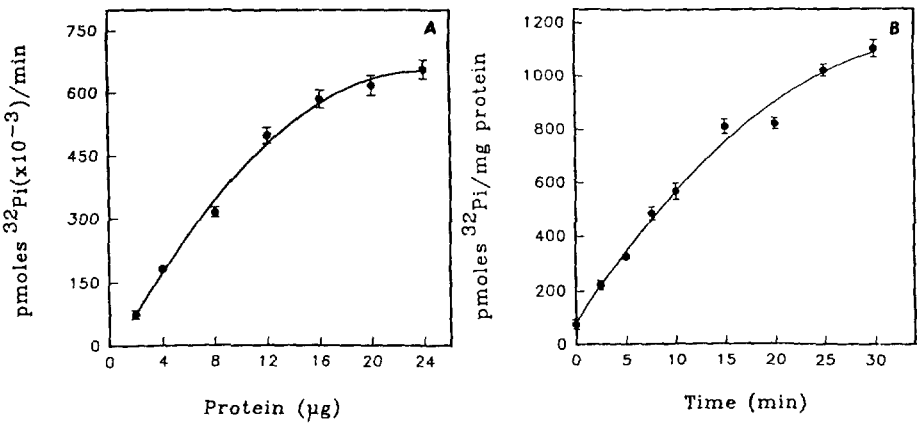

Figure 3. Influence of increasing concentrations of active chromatin proteins (A) and incubation time (B) on the activity of active chromatin protein tyrosine kinases.

The reaction for panel A consisted of PGT (100 µg/ml), varying concentrations of active chromatin protein (0 - 24 µg), cofactors, and 10 µM [γ-^{32}P]ATP as described in "Materials and Methods" . The reaction conditions for the panel B were the same as panel A, except 24 µg active chromatin protein was used and incubation was carried for different time intervals. Each value is a mean of 3-4 measurements.

incubation time (Fig. 3-4). A Lineweaver-Burk plot revealed an apparent Km of 50 µg/ml and Vmax of 45 pmol/min for active chromatin tyrosine kinase(s) (Fig. 4).

Active chromatin tyrosine kinase(s) mediated endogenous protein tyrosine phosphorylation: Western blotting using antiphosphotyrosine antibody revealed several

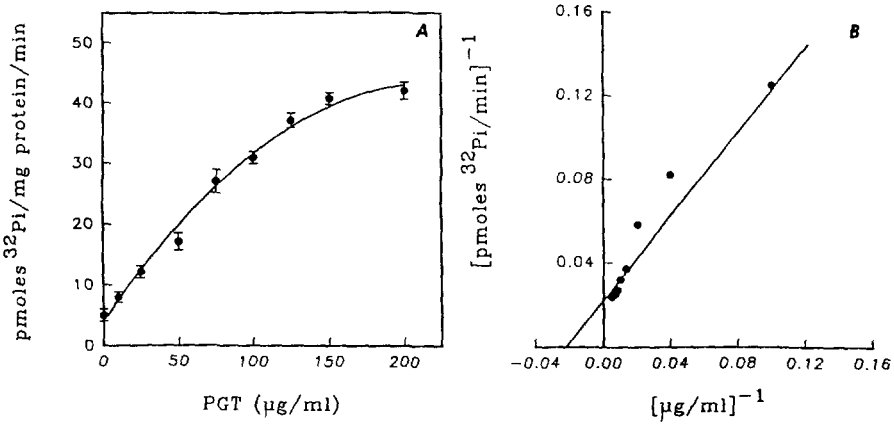

Figure 4. Activity of active chromatin protein tyrosine kinases as a function of substrate (PGT) concentration. (A) Protein tyrosine kinase actvity in the active chromatin (24 µg protein) was measured using 10 µM [γ-^{32}P]ATP, cofactors and varying concentrations of PGT (0-200 µg/ml) as described in "Materials and Methods" . Each value is a mean of 3-4 measurements ± standard deviation. (B) Lineweaver Burk plot showing the apparent Km (50 µg/ml) and Vmax (45 pmol/min) for the phosphorylation of PGT by active chromatin protein tyrosine kinases.

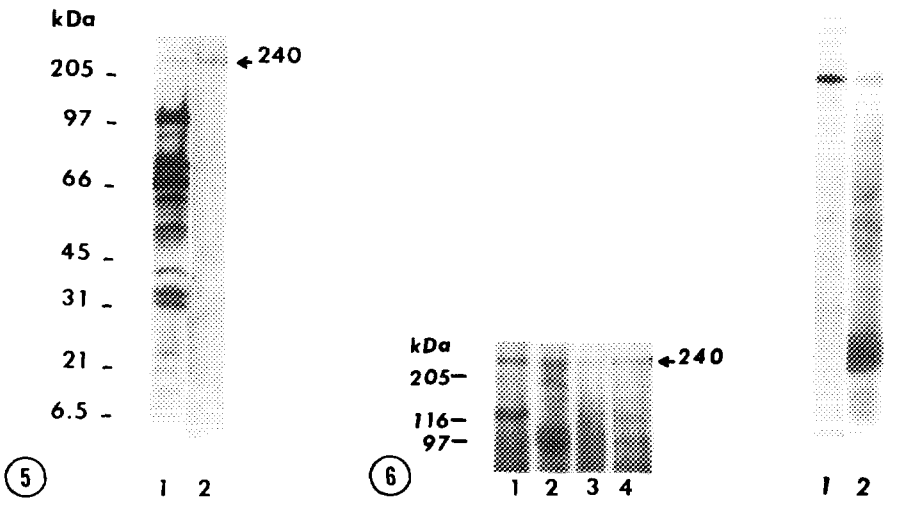

Figure 5. Tyrosine phosphoproteins of the active chromatin.
Endogenous proteins of active chromatin (250 µg) were immunoprecipitated with antiphosphotyrosine antibodies, resolved on SDS-PAGE (7-14%)and transferred to PVDF membrane. The membrane was probed with anti-phosphotyrosine antibody (lane 1) and anti-RNA pol II antibody (lane 2). The molecular weight of the large subunit of RNA pol II (240) is indicated.

Figure 6. Tyrosine phosphorylation of the 240 kDa proteins by AC-PTKs.
Left panel: Endogenous phosphorylation of active chromatin proteins (250 µg) was carried out using 10 µM [γ-^{32}P]ATP and cofactors as described in "Materials and Methods" . Lane 1 = Autoradiograph of labelled proteins resolved on SDS-PAGE; Lane 2 = Autoradiograph of gel treated with alkali; Lane 3 = Autoradiograph of labelled proteins immunoprecipitated with antiphosphotyrosine antibodies; Lane 4 = Western blot of active chromatin proteins probed with antiphosphotyrosine antibody.
Right panel: Purified calf-thymus RNA polymerase IIa (2.5 µg) was phosphorylated using the partially purified active chromatin tyrosine kinases in the presence of 10 µM ATP and cofactors as described in "Materials and Methods" . After the reaction, the samples were centrifuged and the proteins in the supernatant were resolved on SDS-PAGE. The proteins were transferred to PVDF membrane and probed with antiRNA polymerase II antibody (lane 1) and antiphosphotyrosine antibody (lane 2).

proteins ranging from 21 kDa to >200 kDa phosphorylated at tyrosine residues (Fig. 5, lane 1), one of which corresponded to the large subunit (240 kDa) of RNA pol II. Probing the membrane with anti-RNA pol II antibody revealed that the 240 kDa tyrosine containing protein appears to be the large subunit of RNA pol II (Fig. 5, lane 2). The total phosphorylation and tyrosine specific phosphorylation state of the 240 kDa protein of RNA pol II was further analyzed by (a) labeling the active chromatins with ^{32}P and fractionation on SDS-PAGE, (b) resistance to alkali treatment, (c) immunoprecipitation with anti-phospho tyrosine antibody and (d) western blotting with antiphosphotyrosine antibody. The results of all four experiments are presented in figure 6 (left panel). The 240 kDa phosphoprotein (lane 1) was suggested to contain

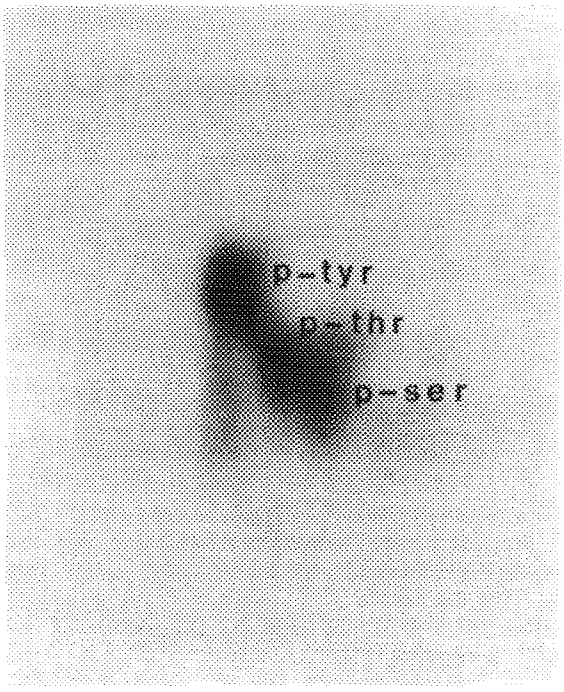

Figure 7. Phosphoaminoacid analysis. The ^{32}P-labeled 66 kDa protein band was excised, hydrolyzed and subjected to 2-dimensional paper chromatography (21). The chromatogram was air dried and autoradiographed. Authentic p-tyr, p-thr and p-ser were visualized with ninhydrin and their positions are indicated with dotted circles.

phospho tyrosine moieties by alkali treatment (lane 2), immunoprecipitation (lane 3) and western blotting (lane 4). Thus, alkali treatment, immunoprecipitation and western blotting by phosphotyrosine antibody suggested that the tyrosine kinases present in the active chromatin phosphorylated the 240 kDa (large subunit of RNA pol II) and several other proteins of active chromatin at tyrosine residues.

Western blotting using phosphotyrosine antibody revealed a single band which matched with that of the large subunit of RNA pol II detected using RNA pol II antibody (Fig. 6, right panel). The autoradiography revealed that the purified RNA pol II phosphorylated by the anti-phosphotyrosine extract from the active chromatin was resistant to alkali treatment (data not shown). These data suggest the presence of protein tyrosine kinase(s) in the active chromatin fraction that phosphorylated the large subunit of RNA polymerase II (240 kDa) at tyrosine residues.

The presence of AC-PTKs was also supported by phosphoamino acid analysis. The heavily phosphorylated 66 kDa band was excised and subjected to phosphoamino acids analysis. Majority of the ^{32}P-label was detected in phosphotyrosine (Fig. 7).

DISCUSSION

The protein tyrosine kinases provide a mechanism for transmission of signal via protein tyrosine phosphorylation from plasma membrane through the cytoplasm to the nucleus. Most of the tyrosine kinases studied so far have been shown to be mainly located in plasma membrane or cytosol (1). We present evidence based on tyrosine phosphorylation of both exogenous and endogenous substrates by active chromatin that protein tyrosine kinase(s) are present in the active chromatin of the nucleus. AC-PTK(s) are able to phosphorylate several active chromatin proteins including the large subunit of RNA pol II at tyrosine residues.

There are several tyrosine containing proteins present in the nuclei as observed in this study and previously (3-8), including transcriptional regulating and DNA binding proteins (for example, RNA pol II, estrogen receptor, androgen receptor, etc.). Since the presence of nuclear tyrosine kinases was not known, the mechanism of phosphorylation of nuclear proteins at tyrosine residues was not clear. It has been considered that cytoplasmic protein tyrosine kinases are responsible for the phosphorylation of nuclear proteins on tyrosine residues (6) in the cytoplasm and which are then translocated into the nucleus. Our data suggest that the AC-PTKs could be responsible for the phosphorylation of the earlier reported tyrosine containing nuclear proteins (3-8) including transcription regulating and DNA binding proteins.

We know that phosphorylation plays important roles in regulating nuclear events including transcription, replication and DNA damage repair (22). Transcription regulating proteins are generally composed of separable DNA-binding and transcription activation domains. Phosphorylation of transcription regulating proteins controls both DNA binding activity and transactivation. In many of the cases phosphorylation inhibits DNA binding. However, studies on the phosphorylation of DNA binding proteins have been focussed on serine and threonine residues only. A recent report revealed that tyrosine phosphorylation of a DNA binding factor is required for the expression of IL4-responsive genes (23). Phosphorylation effects on transactivation are of stimulatory in nature (6). Phosphorylation of general transcription factors and subunits of RNA pol II affects global regulation of transcription. For example, phosphorylation of CTD of RNA pol II at serine and threonine residues by CTD kinases is known to be important for the transformation of initiation complex to the elongation complex during transcription (8). As observed in this study the AC-PTKs catalyzed the tyrosine phosphorylation of RNA pol II both in vitro and in vivo as well as of

several other active chromatin proteins . The possibility of tyrosine phosphorylation of RNA pol II playing an important role in the control of transcriptional activity has been recently reported by us (24). Identification of the specific AC-PTK that phosphorylates RNA pol II or other active chromatin proteins, could provide a better insight in understanding the role of tyrosine phosphorylation in specific nuclear process, is in progress.

Acknowledgment: This research was supported by a grant from the NIH (CA 52584).

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