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Dissociation of the ligand and dephosphorylation of the platelet-derived growth factor receptor

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The ligand-induced phosphorylation of the platelet-derived growth factor (PDGF) receptor was followed at 37°C by a rapid dephosphorylation which was roughly parallel to the down regulation of the ^{125}I-PDGF binding sites. At 4°C, when the ligand-receptor complexes remain associated with the cell surface, the phosphorylated form of the receptor was more stable. However if the ligand was dissociated from the receptor by means of a mild acid wash or a treatment with suramin, the dephosphorylation of the receptor also occurred at a low temperature. These data suggest that, due to the dissociation of the ligand, the kinase activity of the receptor is switched off so that the phosphotyrosine-containing receptors remain exposed to the action of phosphatases that rapidly dephosphorylate them.

Platelet-derived growth factor; Platelet-derived growth factor receptor; Phosphotyrosine; Suramin; Protein kinase; Phosphatase

1. INTRODUCTION

The platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal cells in culture [1]. Specific cell surface PDGF receptors have been identified and the primary structure of murine PDGF receptor has been determined [2]. The ligand-stimulated protein tyrosine kinase activity of the receptor has been studied both in intact cells [3-7] and in 'in vitro' systems [8] and a sequence with homology to other protein kinases has been recognized in the intracellular portion of the receptor [2].

In quiescent cells the PDGF receptor apparently does not contain phosphorylated tyrosine residues. Only upon ligand addition the kinase is activated and the receptor itself becomes phosphorylated in tyrosine. Antibodies against phosphotyrosine (P-tyr) recognize the phosphorylated form of the receptor, and have allowed its purification [8-10]. Before, we have shown that these antibodies can be conveniently employed in the Western blotting technique to monitor the phosphorylation of the receptors in intact cells under different growth conditions [5-7,11].

The phosphorylation state of the receptor, i.e. the amount of P-tyr groups present in the receptor population, is most probably defined in intact cells not only by the kinase activity of the receptor, but also by the activity of endogenous phosphatases [12] which can split off the phosphate from the tyrosine residues.

Here we describe the effect of the dissociation of PDGF on the phosphorylation state of the previously activated receptor molecules.

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2. MATERIALS AND METHODS

2.1. *Cells and growth conditions*

Swiss 3T3 mouse fibroblasts, originally obtained from Dr R. Bravo (Heidelberg), were cultured as reported [5]. For the experiments to be described, cells were plated at a density of 5000/cm^2 in Dulbecco's modified minimal essential medium with 10% fetal calf serum (FCS, from Flow). After 4 days, the medium was replaced with a medium containing 2.5% FCS and monolayers were used after 2 more days.

2.2. *Tyrosine phosphorylation of the PDGF receptor*

Quiescent monolayers were incubated in binding medium [11] in the presence of highly purified human PDGF, with shaking, either at 4°C or at 37°C. At the end of incubation, monolayers

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were extracted and analyzed by immunoblot with P-tyr antibodies [13] as reported [5]. In some cases after autoradiography the 180 kDa band corresponding to the receptor was quantified by counting the ^{125}I of protein A associated with it.

2.3. *Determination of the down regulation of the PDGF binding capacity*

Quiescent monolayers grown in 24 well trays were incubated at 37°C in 0.5 ml of binding medium containing PDGF (30 ng/ml). At the times indicated the trays were transferred to ice, the medium was removed and cells were incubated for 5 min with 20 mM acetic acid, 150 mM NaCl, 0.25% bovine serum albumin (BSA), pH 3.7 [14]. Cells were then repeatedly washed with binding medium and incubated for 2 more h at 4°C with shaking in the presence of ^{125}I-PDGF (53 000 cpm/ng, 6 ng/ml). Non-specific binding (generally about 15% of the total) was determined by adding an excess of partially purified PDGF together with iodinated PDGF. At the end of incubation, the cell associated radioactivity was determined according to Heldin [15].

2.4. *^{125}I-PDGF*

Highly purified PDGF was prepared from outdated human platelets by chromatography on carboxymethylcellulose, Blue Sepharose, Biogel P 50 and HPLC [16]. The final PDGF preparation (ED_{50} for [^3H]thymidine incorporation = 3 ng) was iodinated by the chloramine T method [15] with $Na^{125}I$ from Amersham.

3. RESULTS AND DISCUSSION

Stimulation of intact quiescent Swiss 3T3 fibroblasts with PDGF induces the phosphorylation of the PDGF receptor on tyrosine residues. At 37°C, however, receptor phosphorylation is rapidly followed by its dephosphorylation. Moreover at this temperature the incubation of cells with PDGF also brings about the reduction of the binding capacity for ^{125}I-PDGF [17]. As shown in fig.1, the decrement of the phosphorylated band is roughly parallel to the down regulation of PDGF binding sites. Thus, although as shown previously, dephosphorylation also takes place in the presence of inhibitors of internalization [5], nevertheless under physiological conditions internalization and dephosphorylation of the receptor appear to occur concurrently following stimulation with the ligand.

At 4°C the rate of phosphorylation is slower but the phosphorylated receptor is more stable. Moreover at this temperature internalization does not take place and the PDGF-receptor complexes remain associated with the cell surface. Under this condition either dephosphorylation does not occur or the phosphorylation state of the receptor is a dynamic one, in which the kinase activity of the

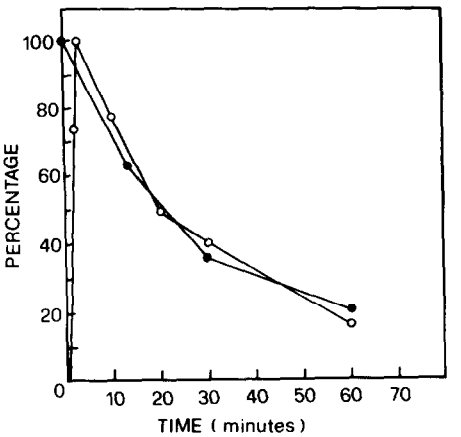

Fig.1. Tyrosine phosphorylation and down regulation of PDGF receptor at 37°C. Receptor phosphorylation (○): quiescent Swiss 3T3 fibroblasts were stimulated with pure hPDGF (30 ng/ml) at 37°C. At the different times, total protein was extracted, fractionated by SDS-PAGE, blotted and immunodecorated with P-tyr antibodies and ^{125}I-protein A. The radioactivity associated with the 180 kDa band corresponding to the PDGF receptor was counted and expressed as a percentage of the maximum. ^{125}I-PDGF bound (●): cells were exposed to hPDGF as above. At different times the cells were washed with acid, exposed for 2 h at 4°C to ^{125}I-PDGF with or without excess of unlabelled PDGF (for determination of aspecific binding). Radioactivity associated with the cells was then determined and specific binding expressed as a percentage of the initial one.

ligand-receptor complexes is counterbalanced by the action of phosphatases.

We have thus analyzed the effect of the dissociation of the ligand from the activated, cell surface-

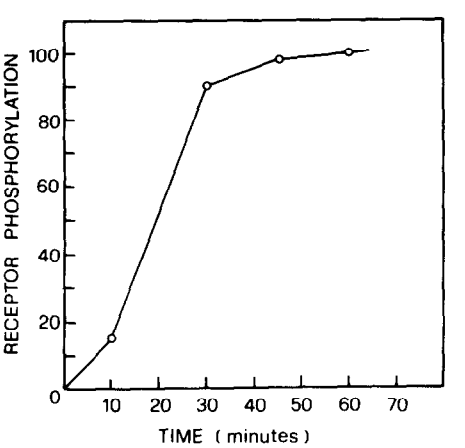

Fig.2. Tyrosine phosphorylation of PDGF receptor at 4°C. Cells were stimulated at 4°C and analyzed for receptor phosphorylation as in fig.1.

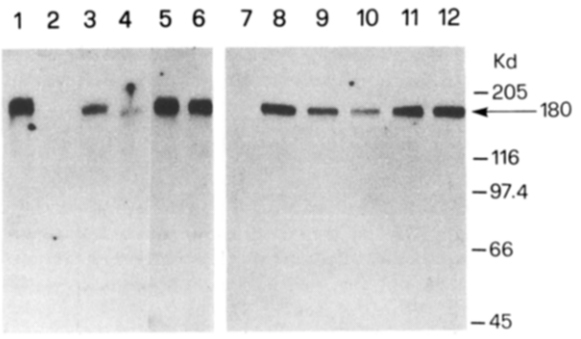

Fig.3. Dephosphorylation of the PDGF receptor induced by treatment with acid and with suramin. Quiescent monolayers were maintained for 30 min at 4°C with no additions (lanes 2 and 7) or with PDGF (lanes 1 and 8). In lanes 3 and 4 cells were exposed to PDGF for 30 min, then, after medium removal, cells were washed for 5 and 10 min, respectively, with 20 mM acetic acid, 150 mM NaCl, 0.25% BSA at 0°C and then analyzed. In lane 5 cells exposed to PDGF for 30 min were then exposed for 10 min to binding medium alone. In lane 6 cells treated as in lane 4 were reexposed to PDGF for 30 min at 4°C. In lanes 9, 10 and 11 cells were treated for 30 min with PDGF, then, after medium removal, cells were exposed for 30 min to binding medium alone (lane 11) or with suramin for 10 min (lane 9) and 30 min (lane 10). In lane 12 cells treated as in lane 10, were carefully washed and then reexposed to PDGF for 30 min at 4°C.

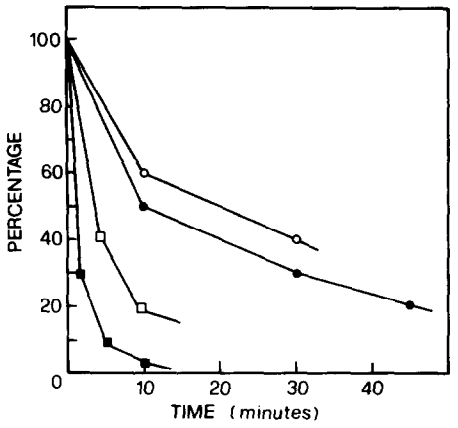

Fig.4. Effect of the treatment with acid and with suramin on the dissociation of prebound PDGF and dephosphorylation or the PDGF receptor. Dissociation of prebound PDGF (●,■) was determined as follows: monolayers were incubated for 2 h in ^{125}I-PDGF (53 000 cpm/ng, 6 ng/ml) at 4°C. Then they were washed and at time zero transferred either in the acid medium in ice (■) or in binding medium containing 1 mM suramin at 4°C (●). At the times indicated the radioactivity associated with the cells was determined and expressed as percentage of the initial one. Parallel samples with unlabelled PDGF in addition to iodinated PDGF were also run for determination of aspecific binding. Dephosphorylation of PDGF receptor (○,□). The data refer to the experiment of fig.3. The intensity of the 180 kDa band corresponding to PDGF receptor was quantified and expressed as percentage of the initial one. Effect of acid treatment (□), lanes 1, 3, 4 of fig.3; effect of suramin (○), lanes 8, 9, 10.

associated receptors on their phosphorylation state. Quiescent Swiss 3T3 fibroblasts were incubated at 4°C with PDGF for 30 min, afterwards the medium was removed and the monolayers were washed with a mild acidic solution according to Bowen-Pope et al. [14] to dissociate the ligand associated with the cell surface. After 5 and 10 min, the total cell protein was analyzed for P-tyr containing protein by Western blotting with P-tyr antibodies. Fig.3 shows that the acid wash caused the rapid dephosphorylation of the receptor (lanes 1–4). When PDGF-treated, acid washed cells were reexposed to PDGF, the receptors became phosphorylated again, indicating that this treatment did not affect the functions of the receptor molecules (lane 6).

Moreover the effect of the acid treatment appears to be really due to the removal of the ligand and not to possible side effects on phosphatases or kinase activities mediated by a shift in intracellular pH: in fact, treatment with acid of NIH 3T3 fibroblasts transformed by Abelson murine leukemia virus did not modify the phosphorylation pattern of P-tyr containing intracellular proteins (fig.5).

Suramin, a heterocyclic polyanionic compound prevents PDGF interaction with its receptor and causes the dissociation of PDGF from the ligand-receptor complex [18]. PDGF-stimulated cells were thus exposed to 1 mM suramin for 10 and 30 min. Fig.3 (lanes 8–10) shows that this treatment caused the dephosphorylation of the receptor, although with slower kinetics than the acid wash.

Incubation with suramin did not affect the activity of the receptors: when, after accurate washing, the cells were reexposed to PDGF, receptor phosphorylation took place again (lane 12).

In parallel experiments the dissociation of prebound ^{125}I-PDGF was monitored: the cells were incubated for 2 h at 4°C with ^{125}I-PDGF. Then the medium was removed and the cells were incubated either with acid or in binding medium with 1 mM suramin. At different times monolayers were analyzed for cell-associated ^{125}I radioactivity. The

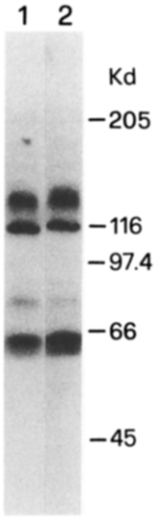

Fig.5. Effect of the acid treatment on P-tyr containing protein of cells transformed by Abelson murine leukemia virus. NIH 3T3 fibroblasts transformed by AMuLV were analyzed by Western blotting with P-tyr antibodies after treatment with acid (as described in fig.3, lane 2), or without any treatment (lane 1).

data obtained are reported in fig.4 together with the data on receptor phosphorylation which were quantified from the experiments of fig.3.

The results indicate that both treatments dissociate the cell surface associated PDGF, although with different kinetics. In both cases dissociation was rapidly followed by receptor dephosphorylation.

These observations suggest that, when the ligand is detached from the receptors, these molecules are dephosphorylated by endogenous phosphatases whose activity prevails over that of the kinase, most probably switched off upon ligand removal.

Thus, as suggested above, the stable phosphorylation of the PDGF receptor observed in 3T3 fibroblasts at 4°C appears to be the result of a balance between the kinase activity of the receptor and the activity of phosphatases. Alternatively, at 4°C in the receptor molecules associated with the ligand, the P-tyr groups could be protected against the action of phosphatases by some sort of conformational modification depending on the association with the ligand.

Moreover, we have reported previously an analogous effect of ligand dissociation on the EGF receptor in A_{431} cells [11]. It appears therefore that kinase activity is uniquely associated with ligand-receptor complexes, and this could be a general feature for growth factor receptors endowed with tyrosine kinase activity. In addition our data indicate that dephosphorylation can also occur without concurrent internalization.

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