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The dephosphorylation characteristics of the receptors for epidermal growth factor and platelet-derived growth factor in Swiss 3T3 cell membranes suggest differential regulation of receptor signalling by endogenous protein-tyrosine phosphatases

Frank-D. Böhmer*, Sylvia-Annette Böhmer*, Carl-Henrik Heldin

Ludwig Institute for Cancer Research, Box 595, Biomedical Center, S-75124 Uppsala, Sweden

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Comparison of the phosphotyrosine-specific dephosphorylation of the autophosphorylated receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) in Swiss 3T3 cell membranes by the endogenous phosphatases revealed striking differences. EGF receptor dephosphorylation was clearly faster than PDGF receptor dephosphorylation and strongly inhibited by Triton X-100 and octylglucoside, whereas PDGF receptor dephosphorylation was to a lesser extent detergent-susceptible. PDGF receptor dephosphorylation was effectively inhibited by phenylarsineoxide, protamine and poly-lysine and partially by *N*-ethylmaleinimide, whereas EGF receptor dephosphorylation was not affected by these agents. We suggest that these differences in dephosphorylation of EGF and PDGF receptors are due to their differential interaction with membrane-associated protein-tyrosine phosphatases and important for differential regulation of receptor signalling.

Epidermal growth factor receptor; Platelet-derived growth factor receptor; Dephosphorylation; Protein-tyrosine phosphatase; Fibroblast membrane (Swiss 3T3)

1. INTRODUCTION

The autophosphorylation of growth factor receptor tyrosine kinases generates binding sites for Src-homology 2 (SH2) domains of downstream signalling molecules and is therefore crucial for tyrosine kinase receptor signalling (for review see [1]). It is likely that regulation of receptor tyrosine phosphorylation by dephosphorylation of the autophosphorylated receptors is important for the regulation of tyrosine kinase receptor signalling. Much attention has recently been focused on the identification and characterisation of protein phosphotyrosine phosphatases (for review see [2]). Various PTPases have been shown to dephosphorylate purified autophosphorylated growth factor receptors in vitro [3–5]. Alternatively, the possible function of certain PTPases in the regulation of growth factor receptor activity has been studied by transfection of cells with PTPase genes [6,7] and subsequent analysis of receptor function. It is not known which PTPase species physiologically interact with the various tyrosine kinase receptors and to what extent the receptor-directed dephosphorylation might be specific. We, therefore, investigated the dephosphorylation of two growth factor receptors in situ in their physiological environment, i.e. the plasma membrane of non-transfected cells. Various parameters of the dephosphorylation of both receptors by the endogenous PTPases were found to be clearly different, pointing to a differential regulation of the different tyrosine kinase receptors by dephosphorylation in the same cell.

*Corresponding authors. F.-D. Böhmer, Max-Planck Society, Research group 'Growth factor signal transduction' and S.-A. Böhmer, Institut für Biochemie, Friedrich-Schiller-Universität Jena, D 07743 Jena, Löbderstr. 3, Germany. Fax: (49) (3641) 82 24295.

Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, receptor for EGF; MES, 2-morpholinoethanesulfonic acid; PDGF, platelet-derived growth factor; PDGFR, receptor for PDGF; PHMB, *p*-hydroxymercurybenzoic acid; PTPase, protein-tyrosine phosphatase; SDS, sodium dodecylsulfate.

2. MATERIALS AND METHODS

2.1. *Materials*

A peptide-specific antiserum (15E) against the EGF receptor was generously provided by W.J. Gullick (London). Antiserum DIG1 was raised against a peptide corresponding to amino acid residues 1,075–1,089 in the human PDGF α-receptor but recognised PDGF α- and β-receptor equally well (L.J. Gonez, unpublished data). For receptor detection in immunoblots, the antibodies were used in the following concentrations: Anti-PDGF receptor antiserum DIG1, 1/1000; anti-EGF receptor antiserum 15E, 1/50.

2.2. *Cells and membrane preparation*

Swiss 3T3 cells were cultured in Dulbecco's modified Eagle medium (Gibco/Bethesda Research Laboratories, Gaithersburg, MD) supplemented with 10% foetal calf serum, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 IU/ml). Membranes were prepared from confluent roller cultures according to the method of Thom et al. [8].

2.3. *Measurement of receptor autophosphorylation and dephosphorylation*

For measurement of receptor autophosphorylation and dephosphorylation, membranes (10 μg protein per assay) were incubated in the presence of 1.2 μg/ml EGF or 2 μg/ml PDGF, 50 mM HEPES (pH 7.5) and 3 mM $MnCl_2$ (final concentrations) in a volume of 40 μl for 20 min on ice. In order to test the effects of various compounds on the autophosphorylation, these were added in a volume of 5 μl 10 min after addition of the growth factor. Phosphorylation was initiated by addition of [γ-^{32}P]ATP (3–5 μCi, final concentration 2 μM) and terminated after 2 min by addition of 10 μl of 6-fold concentrated SDS-PAGE sample buffer. The samples were subjected SDS-PAGE according to the method of Blobel and Dobberstein [9] using 10% acrylamide gels and subjected to autoradiographic analysis. To monitor dephosphorylation, the phosphorylation reaction was quenched after 2 min by addition of unlabelled ATP (pH 7.5, 1 mM final concentration) or EDTA (pH 7.5, 10 mM final concentration). In some experiments the phosphorylation was quenched by addition of hexokinase (Boehringer, Mannheim, 0.2 U/assay) and D-glucose (1 mM, final concentration). After quenching of the phosphorylation reaction, the tubes were shifted to the desired temperature, dephosphorylation was allowed for the indicated time and then abrogated by addition of concentrated SDS-PAGE sample buffer (see above). Standard conditions for effector tests were dephosphorylation for 5 min and 30 min at 15°C for EGF receptor and PDGF receptor, respectively. Vanadate stock solutions were prepared as described [10]. For measuring pH-dependence of the dephosphorylation, phosphorylation was performed in a volume of 25 μl with 20 mM HEPES (pH 7.5) as the buffer. Then, unlabelled ATP was added in another 25 μl of 0.3 M HEPES (pH 6.5–8.5) or 0.3 M MES (pH 5.5 and 6.0) and the dephosphorylation was monitored.

2.4. *Phosphoamino acid analysis*

Phosphoamino acid analysis was performed after immunoblotting as described by Kamps and Sefton [11].

2.5. *Quantification of autoradiograms*

For quantification of radioactivity in electrophoresis gels or thin layer plates, a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) with the program ImageQuant was used according to the instructions of the manufacturer. To obtain autoradiograms, objects were exposed to X-ray film (Fuji RX) with intensifying screen (Dupont Cronex Lightning Plus) at −70°C.

3. RESULTS

3.1. *Receptor dephosphorylation kinetics*

The receptors for EGF and PDGF in membranes isolated from Swiss 3T3 cells were activated with saturating concentrations of growth factors and autophosphorylation was allowed in the presence of [γ-^{32}P]ATP (Fig. 1, inset). Radioactive electrophoretic bands corresponding to the respective autophosphorylated receptors were identified by ligand-stimulated incorporation of radioactivity, molecular mass and immunoblotting with receptor-specific antibodies. The extent of receptor phosphorylation was quantified by autoradiographic analysis with the help of a PhosphorImager. Phosphoamino acid analysis revealed that the autophosphorylated EGF and PDGF receptors contained 92 and 89% phosphotyrosine and 8 and 11% phosphoserine, respectively; phosphothreonine was below the detection limit.

The dephosphorylation kinetics as determined when

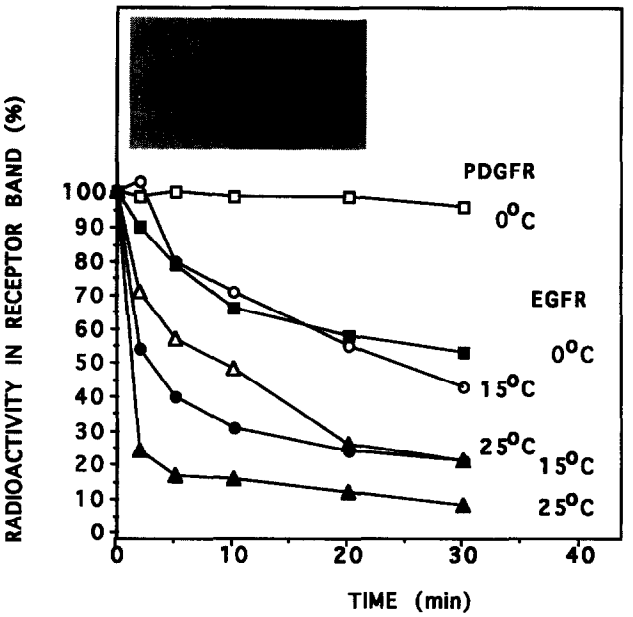

Fig. 1. Time-course of receptor dephosphorylation. Membranes (10 μg protein) isolated from confluent Swiss 3T3 cells were incubated with saturating amounts of growth factors as indicated and phosphorylated with [γ-^{32}P]ATP. The phosphorylation was quenched by addition of unlabelled ATP and the dephosphorylation was monitored at the indicated temperatures. At the respective time-points the reaction was abrogated by addition of SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE and the relative radioactivity in the receptor bands was quantitated with a PhosphorImager. *Inset*: receptor autophosphorylation at start of dephosphorylation experiment (overnight autoradiography).

the kinase reaction was quenched with unlabelled ATP and dephosphorylation monitored at different temperatures are depicted for both receptors in Fig. 1. To ensure that the decrease in radioactivity was not caused by receptor degradation, membrane samples were analysed by immunoblotting for the presence of the receptor protein at different time-points during a dephosphorylation experiment at 25°C and the receptor contents were found to be essentially unchanged. Phosphoamino acid analysis performed at different time-points of receptor dephosphorylation at 25°C revealed that the decay in phosphotyrosine content in the receptors closely matched the decay in total radioactivity, whereas the content in phosphoserine stayed unchanged. The dephosphorylation affected multiple phosphorylation sites, as judged by monitoring receptor dephosphorylation at the level of tryptic phosphopeptide maps (not shown).

The dephosphorylation was clearly temperature-dependent, i.e. accelerated at higher temperature. When compared at the same temperature, EGF receptors were significantly more rapidly dephosphorylated than PDGF receptors. A clearly faster dephosphorylation of EGF receptors as compared with PDGF receptors was also observed when the phosphorylation reaction was quenched with either EDTA or by depletion of radioac-

tive ATP with the help of an efficient ATP-consuming reaction (not shown). Depending on the assay conditions, the difference in the initial dephosphorylation rate of both receptors was 2–10-fold.

3.2. *pH-Dependence*

The pH-dependence of receptor dephosphorylation revealed optimal dephosphorylation for both receptors between pH 6 and 6.5 with relatively little change in the activity between pH 5.5 and 7. Between pH 7 and 8, the dephosphorylating activity decreased significantly.

3.3. *Effector studies*

Various known effectors of PTPases were examined for their effects on the dephosphorylation of EGF and PDGF receptors (Table I). The widely used PTPase inhibitor orthovanadate only partially inhibited the dephosphorylation of both EGF receptors as well as PDGF receptors. In contrast, zinc acetate was an effective blocker of the dephosphorylation of both receptors. *N*-ethylmaleinimide and phenylarsineoxide partially inhibited PDGF receptor dephosphorylation but had little effect on EGF receptor dephosphorylation. P-hydroxymercurybenzoic acid, however, blocked PDGF receptor dephosphorylation completely and EGF receptor dephosphorylation also to a large extent. Dithiothreitol activated the dephosphorylation of both receptors. Poly-lysine and protamine at 25 μg/ml inhibited strongly the PDGF receptor dephosphorylation. Both had almost no effect on the EGF receptor dephosphorylation. Conversely, Triton X-100 and octylglucoside were revealed to be more potent inhibitors of EGF receptor dephosphorylation when directly compared with PDGF receptor dephosphorylation.

For comparison, some of the inhibitors of receptor dephosphorylation were tested for their effects on the receptor autophosphorylation (Table I). Overall, the effects of the agents on kinase activities of the receptors did not correlate with their effects on receptor dephosphorylation. For example, vanadate and Triton X-100 had stimulating effects on the phosphorylation but inhibited dephosphorylation; *N*-ethylmaleinimide potently inhibited the kinase reactions but had only partial effects on PDGF receptor dephosphorylation and no effect on EGF receptor dephosphorylation.

4. DISCUSSION

We suggest that some of the observed characteristics of the dephosphorylation of PDGF and EGF receptors

Table I

Effect of various compounds on receptor dephosphorylation and receptor autophosphorylation in Swiss 3T3 cell membranes

Compound	Concentration	Relative dephosphorylation (% of control)		Relative autophosphorylation (% of control)	
		EGFR	PDGFR	EGFR	PDGFR
Vanadate	0.01 mM	84	67		
	0.1 mM	76	69		
	1 mM	67	76	107	111
Zn-acetate	0.01 mM	80	84		
	0.1 mM	16	27		
	1 mM	0	6	0	0
Phenylarsine oxide	0.1 mM	99	65	70	64
	1 mM	75	39	40	21
	4 mM	64	17	9	5
Iodoacetic acid	1 mM	105	104		
Iodoacetamide	1 mM	101	87		
N-ethylmaleinimide	1 mM	96	73	14	21
	5 mM	92	66		
Dithiothreitol	1 mM	120	151		
	5 mM	132	176		
PHMB	1 mM	39	0		
Spermidine	2 mM	106	106		
Spermine	2 mM	99	78		
$CaCl_2$	2 mM	104	98		
$MgCl_2$	2 mM	107	99		
Heparin	25 μg/ml	100	105		
Protamine	25 μg/ml	83	19	99	49
Poly-lysine	25 μg/ml	95	46	135	60
SDS	1%	0	0		
Triton X-100	1%	49	74	165	122
Octylglucoside	1%	46	70		

Membrane samples were treated with the respective growth factors and the extent of receptor dephosphorylation or receptor autophosphorylation in the absence (100%) or presence of the indicated agents was determined. The figures are means of 3–4 independent experiments.

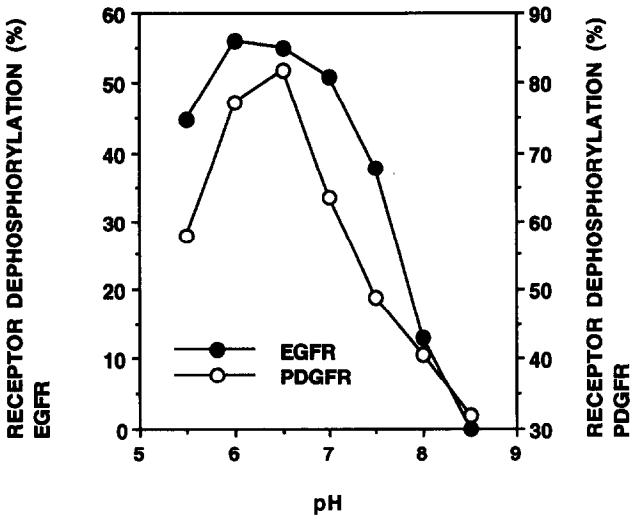

Fig. 2. pH-dependence of receptor dephosphorylation. EGF or PDGF receptors in Swiss 3T3 cell membranes (as indicated) were autophosphorylated at pH 7.5 on ice. Then, the phosphorylation was quenched by addition of unlabelled ATP. Concomitantly, the pH was shifted to the desired value. The figures express the relative extent of dephosphorylation compared to not dephosphorylated samples (0%).

might partially explain previously known characteristics of receptor signalling. So, the relative resistance of PDGF receptors to dephosphorylation could contribute to the more sustained mitogenic effect of PDGF compared to EGF in Swiss 3T3 cells [12]. Relatively small differences in intracellular pH in the range 7–8 were found to have large effects on the receptor dephosphorylation. Intracellular pH-changes in this range might therefore affect receptor signalling activity. For example, the higher intracellular pH observed in growth factor-stimulated cells [13] could support mitogenic signalling by reducing receptor dephosphorylation activity.

In the effector studies, a differential sensitivity of phosphorylating and dephosphorylating activities to various inhibitors was observed. This finding strongly suggests that the observed dephosphorylating activities cannot be intrinsic to the receptors themselves. In keeping with this conclusion, receptor purification by immunoprecipitation or several chromatographic steps removed most of the receptor dephosphorylating activity (not shown). The possibility that the known reversibility of receptor autophosphorylation [14] contributes to the measured decay of radioactivity in the receptors is likewise excluded by these data, in agreement with largely identical results obtained by comparing different methods of quenching of the kinase reaction.

The knowledge of effector susceptibility of receptor dephosphorylation might help to identify the involved elusive PTPases. The relatively low sensitivity of dephosphorylation of both receptors to vanadate versus a high sensitivity to zinc acetate matches previously described inhibitor characteristics of transmembrane-PTPases better than those cytosolic PTPases [4,15,16].

We further observed a differential sensitivity to inhibitors of dephosphorylation of the two different receptors. One obvious explanation for this finding would be the involvement of different PTPases with distinct susceptibilities in the both processes. Alternatively, the lower sensitivity of EGF receptor dephosphorylation to polycations, phenylarsine oxide and some general sulfhydryl-blocking reagents might reflect a sterical hindrance for these reagents to reach the active site of the EGF receptor dephosphorylating PTPase(s). Such a sterical hindrance could be the result of the existence of a tight complex between receptor and PTPase(s) which would prevent access of the reagents. The latter interpretation might explain also the high efficiency of EGF receptor dephosphorylation and in particular, the high susceptibility of EGF receptor dephosphorylation to inhibition of the two receptors is indicated by the results of the inhibition studies.

Previous attempts to identify growth factor receptor PTPases biochemically might have been hampered by the drastic inhibition of receptor dephosphorylation in the presence of detergents, as described in this paper and likewise observed in case of insulin and EGF receptors in rat liver endosomal membranes [17]. Since a denaturing effect of the employed detergents on the involved PTPases is rather unlikely, the detergent susceptibility suggests that the receptor-PTPase interaction is relatively labile and depends on the membrane lipid environment. Thus, future approaches to identify receptor-directed PTPases should aim at stabilising a putative growth factor receptor–PTPase complex.

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