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QUARTERLY

Interferon gamma bound to endothelial cells is phosphorylated by ecto-protein kinases*✪

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The presence of protein kinase activity and its phosphorylated products has been demonstrated on the outer surface of the plasma membrane of endothelial cells. Extracellular phosphorylation was detected by incubation of primary endothelial cells (HUVEC's) and endothelial cell line EA.hy 926 with [γ-^{32}P]ATP. The reaction products were subjected to SDS/PAGE, autoradiography and scanning densitometry. Under the experimental conditions, five proteins with apparent molecular masses of 19, 23, 55, 88, and 110 kDa were prominently phosphorylated in both types of cells. Phosphorylation of the 19 kDa protein was the most rapid reaching maximum after 60 s and then the protein became dephosphorylated. Ecto-protein kinases responsible for the surface labeling of membrane proteins were characterized by using (a) protein kinase C inhibitors: K-252b, chelerythrine chloride, and [Ala113] myelin basic protein (104–118), (b) protein kinase A inhibitor Kemptide 8334, and (c) casein kinase II inhibitor 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB). Stimulation of endothelial cells with tumor necrosis factor α (TNFα) and interferon γ (IFNγ) is associated with 20–80% reduction of extracellular phosphorylation of all membrane proteins. IFNγ bound to membrane receptors becomes rapidly phosphorylated. Only in the case of IFNγ it was associated with the appearance of a strongly phosphorylated band of 17 kDa corresponding to IFNγ itself. Phosphorylation of this 17 kDa exogenous substrate was prevented by an ecto-kinase inhibitor K-252b. The existence of ecto-phosphoprotein phosphatase activity in endothelial cells was evidenced by testing the effect of microcystin LR – a membrane impermeable reagent that inhibits both PP-1 and PP-2a phosphoprotein phosphatases. The extent of phosphorylation of 19

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Abbreviations: HUVEC, human umbilical vein endothelial cells; DRB, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole; IFNγ, interferon γ; TNFα, tumor necrosis factor α; CK1, CK2, casein kinase (I, II); PKA, cAMP dependent protein kinase; PKC, protein kinase C.

kDa and 110 kDa phosphoproteins significantly increased in the presence of microcystin. Our results suggest the presence of at least two ecto-kinase activities on endothelial cells that may play a significant role(s) in the regulation of cytokines function.

Pulse treatment of human vascular endothelial cells with TNFα and IFNγ results in reversible inhibition of growth and proliferation of cells [1]. Both cytokines synergize in a variety of biological responses, including the induction of cytokine, cell adhesion, and inducible nitrous oxide synthase gene expression. Typically, the synergistic effect on gene expression is due to the independent activation of nuclear factor kappaB (NF-κB) by TNFα and activation of signal transducers and activators of transcription (STATs) or IFN-regulatory factor 1 by IFNs, allowing these transcription factors to bind to their unique promoter sites.

Here we report that the earliest events induced by both cytokines in endothelial cells can be already detected on the surface of endothelial cells and are associated with a reduction of extracellular protein phosphorylation by membrane ecto-kinases. Protein kinase activities utilizing extracellular ATP have been described on the surface of different cells, including neurones, neutrophils, fibroblasts and platelets [2-12]. However, the physiological significance of extracellular phosphorylation of proteins is not yet understood. In cells which store ATP within secretory vesicles and release it upon cell stimulation, extracellular protein phosphorylation systems may play an important role in cellular activation and intercellular communication [13]. The same mechanism may be suggested for endothelial cells being in close contact with the secretory blood cells and thus exposed to ATP released during different activation processes. A number of extracellular proteins, for example fibrinogen, fibronectin and vitronectin, that can be used by endothelial cell receptors as adhesive ligands, are phosphorylated by ecto-kinases [14]. Such extracellular modification in the case of vitronectin significantly enhances adhesion of endothelial cells probably due to an increased affinity toward its receptor, a glycoprotein complex $\alpha v\beta_3$ [15].

In the present work we describe membrane proteins of endothelial cells that become phosphorylated by ecto-protein kinases utilizing extracellular ATP. Then we attempt to characterize the type of ectokinases in endothelial cells using selective kinase inhibitors. Finally, we evaluate the effect of TNFα and IFNγ on extracellular phosphorylation on endothelial cells.

MATERIALS AND METHODS

Materials. Protein kinase inhibitors, chelerythrine chloride, K-252b, [Ala113]myelin basic protein (104-118), and 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) were purchased from Alexis Corporation. Kemptide 8334 was from LC-Laboratories. Protein phosphatase inhibitor microcystin-LR was obtained from Calbiochem. All reagents for tissue culture were from GIBCO. Cytokines, INFγ and TNFα were purchased from Sigma. [γ-^{32}P]ATP was supplied by Center of Macromolecular and Molecular Research (PAS, Łódź). All reagents for PAGE were from Bio-Rad.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords by collagenase treatment. Cells were grown in gelatine-coated tissue culture flasks and were maintained at confluence in RPMI 1640 medium supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), heparin (90 μg/ml), L-glutamine (1 mM), sodium bicarbonate (2 mg/ml), 20% foetal calf serum and epidermal growth factor (40 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. Primary cultures were harvested at confluence with

trypsin/EDTA, and passaged into gelatine coated dishes. All experiments were performed with cells grown on 48 wells plates for 1 or 2 days. Then cultures were incubated overnight in fresh serum-free medium.

Human endothelial cell line EA.hy 926, derived by fusion of human umbilical vein endothelial cells with continuous human lung carcinoma cell line A549, was obtained as a gift from Professor Cora-Jean S. Edgell (Pathology Department, University of North Carolina at Chapel Hill, U.S.A.). EA.hy 926 cells closely resemble HUVEC cells and maintain characteristics of differential endothelium. The cells were cultured in DMEM with high glucose, supplemented with 10% foetal calf serum, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), and antibiotics in a 90–95% humidified atmosphere of 5% CO_2 at 37°C.

Phosphorylation assay conditions. Phosphorylation reactions were performed with intact cells as described previously [10]. In brief, medium from each well was removed and intact cells were gently rinsed with Krebs-Ringer buffer (pH 7.4) prewarmed to 37°C. All reactions were carried out in a 37°C. To start the reaction, label was added as 10 μCi of [γ-^{32}P]ATP. Final concentration of ATP was 0.1 μM. Incubation times with [γ-^{32}P]ATP from 10 s up to 1 h were used. Reactions were terminated by the addition of Laemmli SDS stop solution and heating at 95°C for 5 min. Reaction products were subjected to SDS/PAGE, autoradiography and scanning densitometry.

SDS-gel electrophoresis. Samples were separated by polyacrylamide gel electrophoresis (8–18% linear gradient) under reducing conditions according to the method of Laemmli [16]. Gels were stained with Coomassie Brilliant Blue, dried, then phosphorylated reaction products were analysed by autoradiography and scanning densitometry using the programme Image Master VDS 3.0 from Pharmacia Biotech (Austria).

RESULTS

Protein phosphorylation at the surface of endothelial cells

Endothelium, being in close contact with the secretory blood cells, is exposed to ATP released during activation processes. To show whether extracellular ATP is utilised by endothelial ecto-protein kinases resulting in the phosphorylation of specific membrane proteins, assays of surface phosphorylation were carried out. Intact endothelial cells isolated from human umbilical vein were incubated for different intervals with exogenously added [γ-^{32}P]ATP at final concentration 0.1 μM and the reaction was terminated by the addition of the SDS-stop solution. The reaction products were subjected to SDS/PAGE, autoradiography and scanning densitometry (Fig. 1A). According to the criteria required for ecto-phosphorylation [12] five major surface-located proteins were identified as endogenous substrates for ecto-protein kinases in primary endothelial cells. They showed molecular mass of 110, 88, 55, 23 and 19 kDa. The phosphorylation of most of the endogenous substrate proteins in HUVEC's was time-dependent and reached the maximum after 30 min (not shown). Only 19 kDa differed in phosphorylation kinetics in HUVEC's. Its phosphorylation was very rapid, detectable already after 10 s and reached the maximum at 60 s (not shown). This substrate was also rapidly dephosphorylated. When immortalised endothelial cell line EA.hy 926 was tested under the same conditions, an additional membrane protein of 35 kDa was phosphorylated (Fig. 1B). Phosphorylation of 19 kDa protein was detected already after 10 s, but its kinetics was slower as compared to primary cells. For all subsequent experiments homogenous population of endothelial cell line EA.hy 926 and incubation for 10 min with [γ-^{32}P]ATP were selected as the standard phosphorylating condition. Labeling of 110, 88, 55, 35,

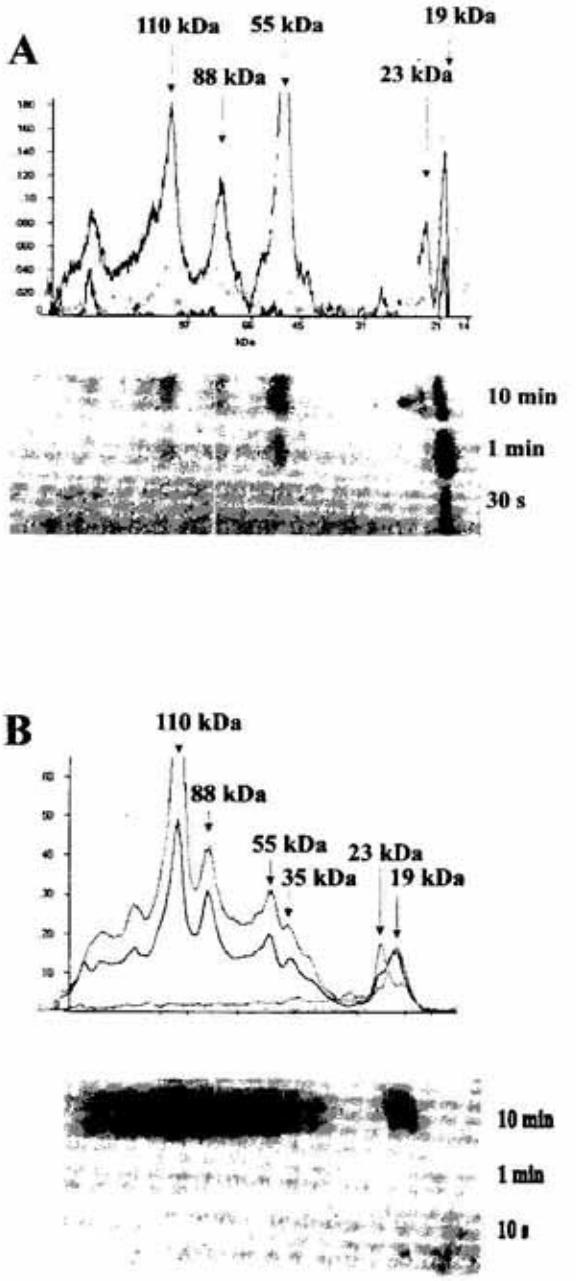

Figure 1. Endogenous protein substrates of ecto-kinase activity in intact HUVEC's and EA. hy 926 cell line.

HUVEC's (Panel A) and endothelial cell line EA.hy 926 (Panel B) were incubated with [γ-^{32}P]ATP and aliquots withdrawn at different time intervals. Panel C shows autoradiogram of proteins labeled in EA.hy 926 incubated for 10 min with [γ-^{32}P]ATP in the absence (lane a) or presence of apyrase (lane b), trypsin (lane c), and nonlabeled ATP (lane d).

23 and 19 kDa was completely prevented by adding apyrase to the medium (Fig. 1C), indicating that phosphorylation of these proteins occurs only in ecto-domains. Furthermore, labeling of the protein substrates of ecto-protein kinase was not affected by the addition of 10000-fold excess (1 mM) of unlabeled P_i to the cells but it was prevented by treatment of the cells with 0.01% trypsin, and was blocked by 1 mM ATP. The complete inhibition of the phosphorylation of endogenous proteins by apyrase and trypsin (which do not cross the plasma membrane) provided further evidence that it could occur only extracellularly.

Characterisation of ecto-protein kinase acting on endothelial cells

To characterise ecto-protein kinases involved in phosphorylation of extracellularly orientated membrane proteins, intact EA.hy 926 cells were incubated with [γ-^{32}P]ATP in the presence of specific protein kinase inhibitors. As shown in Table 1, incorporation of ^{32}P into membrane proteins was strongly inhibited by protein kinase C inhibitors:

[Ala113]myelin basic protein (104–118), chelerythrine chloride and K-252b. Extracellular phosphorylation of endogenous phosphoproteins was also slightly inhibited by the inhibitor of PKA – Kemptide 8334, and casein kinase II – DRB (not shown). Interestingly, treatment of cells with phorbol 12-myristate-13-acetate (PMA, 1 μM), a specific activator of protein kinase C, had no significant effect on extracellular phosphorylation observed in endothelial cells (not shown).

Phosphorylation of interferon γ by endothelial ecto-protein kinase

Activation of cells with TNFα (1 μg/ml) and INFγ (1 μg/ml) for 30 s significantly reduced phosphorylation of 110, 88 and 23 kDa endogenous substrates for ecto-protein kinase on EA.hy 926 cells (Fig. 2). Unexpectedly, when IFNγ was used, a new phosphoprotein of 17 kDa corresponding to this cytokine appeared on the gel. Phosphorylation of 17 kDa was rapid (30 s) and was accompanied by inhibition of the phosphorylation of all major membrane protein substrates, especially 110 kDa (Fig. 3C). To show the identity of the 17 kDa protein with IFNγ, in the next experiments different amounts of IFNγ were added to EA.hy 926 cells and the extent of extracellular phosphorylation was evaluated under standard conditions. Figure 3A shows a concentration dependence of IFNγ phosphorylation. Adding IFNγ up to 7.6 μg/ml into cell media resulted in an almost complete inhibition of phosphorylation of all membrane phosphoproteins associated with high incorporation of ^{32}P into IFNγ (Fig. 3C). This indicates that IFNγ efficiently competed out all extracellular substrates for ecto-protein kinase present on the surface of endothelial cells. To test to what extent phosphorylated IFNγ remains associated with cell membranes, EA.hy 926 cells were incubated with [γ-^{32}P]ATP in the presence of different concentrations of IFNγ for 10 min (Fig. 3A). Then the supernatant was separated from the cells which were washed twice with Krebs-Ringer buffer and lysed with SDS stop buffer. Electrophoretic separation of IFNγ present in the supernatant and in lysed cells indicates that the majority of phosphorylated IFNγ was released from the cell membrane (Fig. 3B). However, a substantial amount of phosphorylated IFNγ remained bound to its membrane receptor. Phosphorylation of IFNγ by endothelial cells was inhibited in the presence of K-252b at 1 μM final concentration (Fig. 4), which is known to inhibit ecto-protein kinase.

Effect of phosphatase inhibitor on extracellular phosphorylation

The existence of ecto-phosphoprotein phosphatase activity was previously reported on the surface of human blood platelets [17]. To

Table 1. Effect of protein kinase C inhibitors on ecto-kinase activity in EA.hy 926

Molecular mass (kDa)	Inhibition of phosphorylation (%)		
	[Ala113]MBP	Chelerythrine chloride	K-252b
110	55.8 ± 15.3	53.0 ± 11.5	45.6 ± 15.2
88	60.7 ± 19.4	58.6 ± 12.0	68.9 ± 9.8
55	58.0 ± 27.6	25.8 ± 9.2	36.9 ± 6.3
23	31.4 ± 16.1	16.6 ± 4.6	14.0 ± 7.1
19	76.4 ± 19.2	28.9 ± 13.4	12.9 ± 3.0

Cells were incubated with [γ-^{32}P]ATP in the presence of protein kinase C inhibitors: [Ala113]MBP (1 μg/ml), chelerythrine chloride (10 μM), and K-252b (1 μM) for 10 min. Data represent mean value ± S.D. and each value was calculated from at least four determinations.

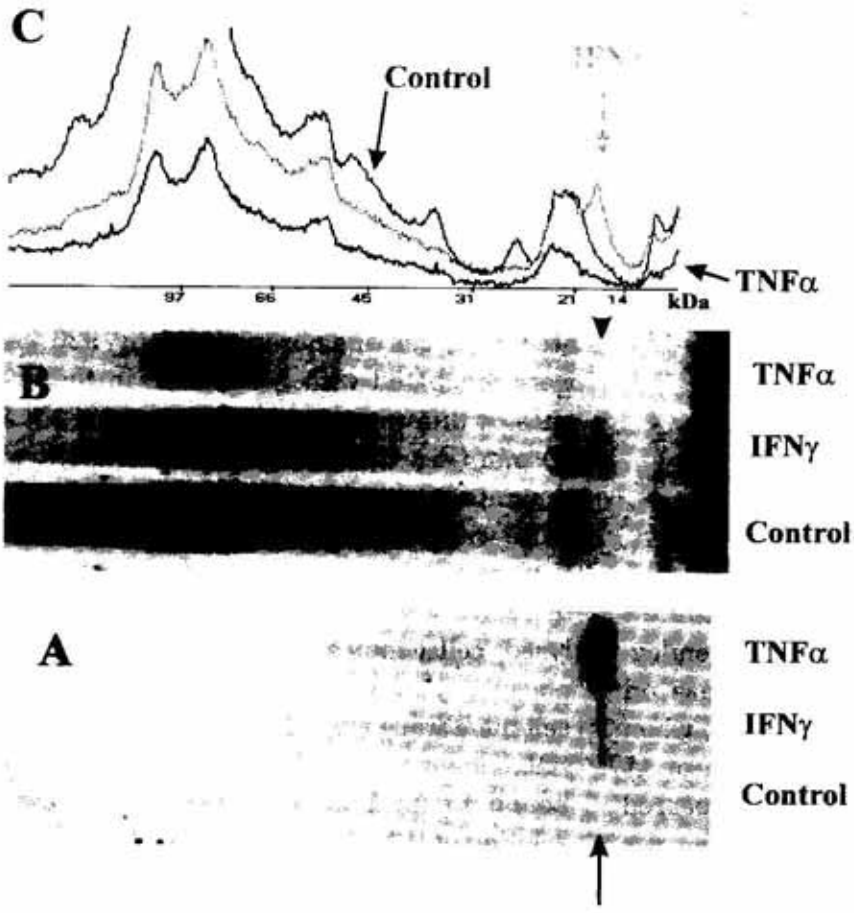

Figure 2. Effect of IFNγ and TNFα on phosphorylation by extracellular kinase.

Cells were incubated with [γ-^{32}P]ATP in the absence or presence of 1 μg/ml of IFNγ and TNFα. Panel A shows Coomassie Blue stained gel indicating the position of both cytokines. Autoradiogram of the same gel (Panel B) and its densitometric scanning (Panel C).

determine whether a surface phosphatase system operates on the studied endothelial cells, we examined the effect of a phosphoprotein phosphatase inhibitor – microcystin LR on surface protein phosphorylation in EA.hy 926 cells. Phosphorylation of 19 and 110 kDa en-

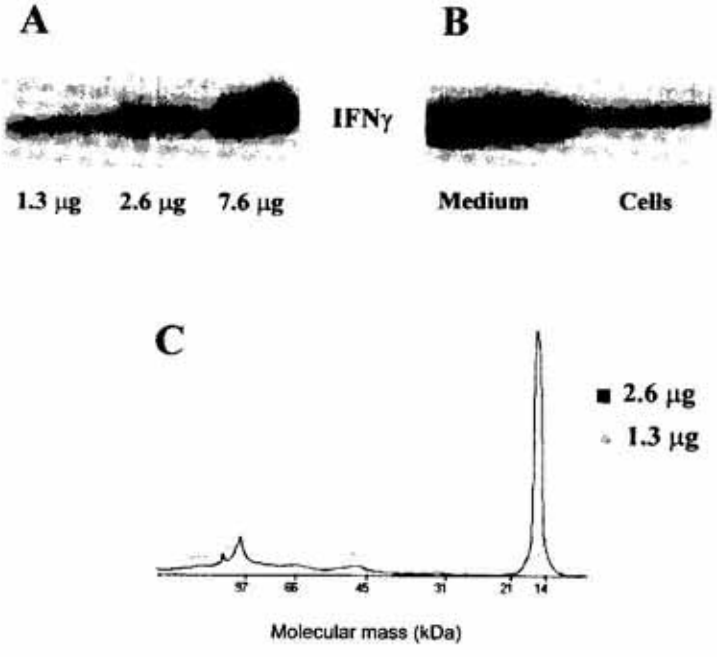

Figure 3. Phosphorylation of IFNγ by ecto-protein kinase in endothelial cells.

EA.hy 926 cells were incubated with [γ-^{32}P]ATP in the presence of different amounts of IFNγ (Panel A). Panel B shows the presence of phosphorylated IFNγ both in cell medium and bound to the cell receptor. Panel C shows scanning profiles obtained from autoradiograms on which the reaction products of two cell suspensions treated with two concentrations of IFNγ (1.3 and 2.6 μg/ml) are separated.

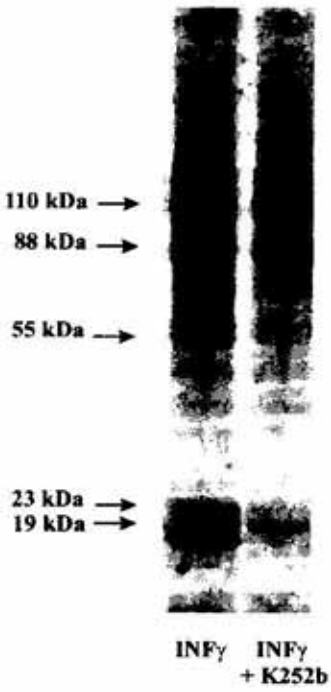

Figure 4. Effect of K-252b on IFNγ phosphorylation by endothelial cells.

Phosphorylation reaction was carried out in the presence of 1 μg/ml of IFNγ and with K-252b added at 1 μM concentration.

dothelial cells surface proteins was significantly enhanced in the presence of microcystin, which does not penetrate the plasma membrane (Fig. 5). An addition of the inhibitor at 1–100 nM concentration to the reaction medium caused a concentration dependent increase in the extent of extracellular phosphorylation of endogenous substrates in endothelial cells.

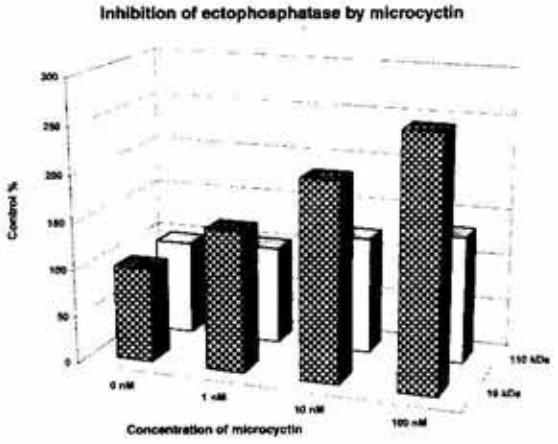

Figure 5. Effect of microcystin on extracellular phosphorylation in EA.hy 926 cells.

Microcystin was added to EA.hy 926 cells 30 s before starting the phosphorylation reaction, carried out as described in Materials and Methods.

DISCUSSION

Protein phosphorylation is a post-translational event mediated by kinases that may have a profound influence on biological activity. Thus, protein phosphorylation is an integral part of regulatory mechanisms in cells, including receptor-mediated signal transduction, second messenger signalling and modulation of gene transcription. Although these phosphorylation reactions occur primarily within the cell, the activity of extracellular kinases has also been implicated in cell-cell communication [9, 11, 18], cell adhesion and other cell functions [19]. In this study, we demonstrated the presence of a protein kinase and phosphorylated products on the outer surface of the plasma membrane of primary human endothelial cells and EA.hy 926 cell line by incubating intact cells with [γ-^{35}P]ATP. The sensitivities of the phosphorylation of endothelial cells to trypsin digestion of cell surface proteins as well as to apyrase were similar to those reported for ecto-protein phosphorylation in other systems [4, 20]. It is conceivable that $^{32}PO_4^{3-}$ released by extracellular ATPases could enter the cell, but its specific activity would be markedly lowered by the presence of 1 mM PO_4^{3-} in the labeling medium. Indeed, increasing [PO_4^{3-}] to 1 mM did not influence ecto-kinase activity, whereas intracellular protein phosphorylation was not detectable when cells were incubated with $^{32}P_i$ for the same period of time (10 min). Further, exogenously added proteins, including MBP, dephospho-alpha-casein and phosvitin, that do not enter the cell were phosphorylated causing a decrease in the phosphorylation of endogenous substrates of ecto-protein kinase (data not shown). Thus, endothelial cells catalysed the transfer of the γ-phosphate of ATP to exogenous proteins

added to the reaction buffer. Finally, phosphorylation was abrogated by the presence of the cell-impermeable ecto-kinase inhibitor K-252b [21] in the culture media of confluent endothelial cells. Based on these criteria we identified major membrane phosphoproteins – the substrates for ecto-protein kinase, which had essentially the same molecular mass in primary endothelial cells and the endothelial cell line EA.hy 926. Ecto-protein kinase activity was almost completely inhibited by the tested inhibitors specific to protein kinase C. Three types of ecto-protein kinases were found on the cell surface: CK1 [22], CK2 [22, 23] and PKA [24]. Since all inhibitors of PKC studied in this work, namely chelerythrine, K-252b, and [Ala113]MBP (104–118) abolished phosphorylation of all major membrane phosphoproteins, it appears that the ecto-protein kinase found on these cells has a protein kinase C activity. Inhibitors of PKA and CK2 show slight effect on the phosphorylation of endogenous proteins, thus PKA and CK2 play only a minor role if any in the extracellular phosphorylation in endothelial cells. The effect of membrane-impermeable phosphatases inhibitor evidenced the presence of an ecto-phosphatase system acting on the surface of EA.hy 926 cells.

In this study we show for the first time that in addition to membrane proteins, biologically active molecules such as IFNγ can be modified by ecto-kinase phosphorylation. Phosphorylation of the exogenous IFNγ was blocked by cell-impermeable ecto-kinase inhibitor K-252b. IFNγ contains several serine and threonine residues which can be potentially phosphorylated [25], but only serine was phosphorylated by kinases released by various cells [26]. However, at present time, it is not known which of the residues became phosphorylated by ecto-kinase in endothelial cells. Although the majority of phosphorylated IFNγ molecules is released from the surface of endothelial cells, there is a small fraction of the cytokine associated with high-affinity membrane receptors. Binding of IFNγ to endothelium appears to be an at least two-phase process. Firstly, IFNγ is sequestered at the surface of endothelial cells by electrostatic interaction between specific basic amino-acid residues and sulphated domains on heparan sulphate (HS), the most abundant endothelial glycosaminoglycan. This interaction is competitively inhibited by heparin, which is structurally related to heparan sulphate and by this mechanism heparin antagonises the activation of a model endothelium by IFN-γ [27]. Secondly, IFNγ associated with HS engages the high-affinity receptor and signal transduction. Whether IFNγ becomes phosphorylated during the first or the second stage of binding to endothelial cells, and what is the physiological significance of such modification is not yet known.

Interestingly, a putative cell growth inhibitor activated by phosphorylation with the same molecular mass and other characteristics as IFNγ was found recently in transformed fibroblasts [28]. Similarly, in ATP-treated cultures of human breast cancer cells, the inhibitory activity was found to be in the molecular mass range of 8–24 kDa [29]. Another example for an inhibitor that is released from the cell in its latent form and activated outside the cell is the transforming growth factor-β.

To summarise, the present study showed that human endothelial cells have an ecto-protein kinase that utilises extracellular ATP to phosphorylate cell surface proteins. This activity is inhibited by peptide substrates specific for PKC and is abolished by specific inhibitors such as K-252b and chelerythrine chloride indicating that ecto-protein kinase acting on the surface of endothelial cells has the catalytic specificity of PKC. Our study demonstrates for the first time that IFNγ can undergo selective phosphorylation by a protein kinase located on the surface of human endothelial cells. Interestingly, only a fraction of phosphorylated IFNγ remains bound to its membrane receptor while the majority of phosphorylated IFNγ can be found in culture medium.

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