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The sulfhydryl reagent N-ethylmaleimide induces hyperphosphorylation on tyrosine residues in the Jurkat T-cell line

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Tyrosine protein kinases have been shown to be functionally involved in regulation of cellular signalling, proliferation and transformation. The activity of tyrosine protein kinases is counterbalanced by phospho tyrosine phosphatases that maintain constitutively low levels of protein phosphotyrosine in most cells.

In this study the effect of N-ethylmaleimide on the protein tyrosine phosphorylation was tested in Jurkat T-cells. Treatment of intact cells for 5-10 mins with 50-100 μM N-ethylmaleimide resulted in a dramatic increase in phosphorylation on tyrosine residues. Phosphoaminoacid analysis revealed an up to ten-fold increase in the content of phosphotyrosine. N-ethylmaleimide blocked the phospho tyrosine phosphatases activity of immunoprecipitated CD45 while in a kinase assay N-ethylmaleimide did not affect the ^{32}P-γ-ATP phosphorylation of substrates. The N-ethylmaleimide-induced hyperphosphorylation was reversed by treatment with 2 mM dithiotreitol. It is concluded that N-ethylmaleimide offers a novel useful tool for identification of substrates for tyrosine protein kinases and for studies on phosphotyrosine-dependent protein interactions.

Tyrosine phosphorylation is an early and essential event in T-cell signalling. Agents that inhibit TPK activity also completely block receptor-mediated T cell activation (1, 2). Two TPKs of the non-receptor src-family have been functionally implicated in the early phases of T cell triggering : p56lck which is pysically linked to the CD4/CD8 molecules (3-5) and p59fyn which is found associated with the TCR/CD3 complex (6, 7).

The overall content of protein phosphotyrosine is dependent on the balance between TPK and PTPase activities. Usually the amount of phosphotyrosine is very low in most cells, suggesting a dominance by PTPases. In T cells the CD45 molecule (for review see (8)) displays a majority of the membrane-associated PTPase activtiy through the cytoplasmic portion of the molecule (9, 10). There are evidence that CD45 functionally regulates the T-cell signalling (11-13). In addition to CD45 several cytosolic PTPases in T-cells have also been identified (14, 15). Their role in the signal transduction or T-cell activation is still poorly understood.

Abbreviations: NEM, N-ethylmaleimide; PAO, phenylarseneoxide, TPK, tyrosine protein kinase; PTPase, phospho tyrosine phosphatase; VO4, sodium orthovanadate.

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In this study it is demonstrated that treatment of intact cells with the sulfhydrylreagent NEM induces a hyperphosphorylation on tyrosine residues. The hyperphosphorylation was seen within minutes after addition of the drug. Analysis of phosphoaminoacids revealed an almost ten-fold increase in the phosphotyrosine content. This dramatic NEM-induced phosphorylation could be reversed by extended treatment with dithiotreitol.

Materials and Methods

Reagents : NEM and PAO (Aldrich Chemie, Steinheim, Germany) were dissolved in DMSO. The anti-phosphotyrosine moAb P-3300 was from Sigma ImmmunoChemicals (St. Louis, MO) and the ^{125}I labelled anti-mouse ab, IM 131, from Amersham (UK).The BSA (Sigma Chemicals, St. Louis, MO) was precleared on a proteinA sepharose column (Pharmacia, Sweden)

Cells : Jurkat, a human T-cell leukemia line was cultured at +37°C in humidified air with 5% CO_2 in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with L-glutamine, antibiotics and 5% (vol/vol) fetal calf serum (Gibco, Paisley, UK).

Anti-P-Tyr immunoblots : Cells at 5-10 x 10^6/ml were preincubated with indicated concentrations of NEM and/or DTT and lysed with an ice-cold buffer containing 50 mM TRIS/HCl pH 8.5, 5mM EDTA, 1% NP-40, 1 mM orthovanadate and 10µg/ml of aprotinin and leupeptin. Aliquots of centrifuged samples were added to boiling SDS sample buffer (v/v, 1:1) (125 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, 1 mM orthovanadate). The samples were separated by SDS-PAGE (10% gels) under reducing conditions and electrophoretically transferred to nitrocellulose filters. Unspecific binding was blocked with 3% BSA in 20 mM Tris/HCl, pH 7.5, NaCl 150 mM, 0.05 % Triton X-100 (3% BSA-TBS) for 2h. Immunoblotting with monoclonal anti-phosphotyrosine in 0,5% BSA-TBS was performed as described (16). The ^{125}I-labelled anti-mouse ab was used at 0.5-2 µCi/ml.

Immunohistochemical staining: Cytocentrifuged Jurkat cells were fixed 5 min in ice-cold methanol and stained with a 1:500 dilution of anti-phosphotyrosine moAb. A FITC-conjugated sheep anti-mouse ab at 1:40 dilution was used as the secondary antibody and samples examined under a Zeiss Axiophot microscope.

Phosphoamino acid analysis : Jurkat cells (3 x 10^6) were labelled with 125 µCi/ml ^{32}P-orthophosphate in 2 ml of phosphate-free MEM medium, 10 mM HEPES, pH 7.2 for 24 h. NEM was added for the last 20 min. Washed samples were lysed with ice-cold 10% TCA. The precipitated proteins were centrifuged 15 000 x g 2 min and the resulting pellets washed x3 with ice-cold acetone. Samples were dried and subjected to acid hydrolysis in 6M HCl at 110°C for 2 h. The phosphoamino-acids were separated on cellulose thin layer plates by twodimensional electrophoresis at pH 1.9 and 3.5 as described (17, 18).

^{32}P-γATP phosphorylation assay: Membranes of 10^8 Jurkat cells were prepared as described (19) with 1 mM sodium orthovanadate present during isolation of the membranes. The pelleted membranes were resuspended in 150 µl 10mM Hepes pH 7.5 and approximately 100 µg to 20 µl of an assay buffer, containing 0.1 % TX-100, 5 mM Mn^{2+}, 5 mM B-ME, 1 mM "cold" ATP and 10µCi of ^{32}P-γATP (Amersham, UK), used. The incubations were ended by addition of 20 µl boiling SDS sample buffer. Radiolabelled proteins were analyzed by SDS-PAGE and autoradiography.

PTPase assay: 3 x 10^7Jurkat cells were lysed with an ice-cold buffer containing 0.2% NP-40, 150 mM NaCl, 1 mM orthovanadate, 20 mM imidazole pH 7.1, 10µg/ml of aprotinin and leupeptin and centrifuged 15 000 x g. To samples, precleared with 20µl anti-mouse agarose (Sigma Chemicals, St. Louis, MO), 10 µl of an anti-CD45 moAb (M701, Dako A/S, Denmark) was added. After one hour CD45 was immunoprecipitated with 50 µl anti-mouse agarose. Immunoprecipitates were washed x2 in lysis buffer, x1 in lysis buffer containing 0.5 M NaCl and x2 in lysis buffer. Assay buffer containing 0.2 % TX-100, 10 mM orthophosphotyrosine, 1 mM EDTA and 100 mM Na-acetate pH 6 was added. Incubation was ended with icecold 20 % TCA. Vortexed vials were kept on ice for 10 min, centrifuged 5 min 15000 x g and free phosphate of the supernatant measured according to the method of Chen et al. (20). The absorbance of the sample taken before incubation at +37°C was used as the reference level.

Results

Treatment of Jurkat T-cells with 100 μM NEM for 20 mins induced a dramatic increase in phosphorylation on tyrosine residues as detected by anti-phosphotyrosine immunoblotting (fig 1A). This NEM-induced hyperphosphorylation occurred within 3 mins and a maximal level was reached by 10-20 mins (fig1A). Dose-dependence of NEM-induced phosphorylation is shown (fig 1B). Treatment with NEM induced phosphorylation on proteins with apparent MWs of 34, 45-46, 49, 54, 55-62, 70, 72-75, 84, 95, 106-110, 120-125 and 140-150 kDa. The effect of NEM on the phosphotyrosine content in Jurkat cells can also be visualized through immunohistochemical staining. NEM-tretated, cytocentrifuged Jurkat cells, stained with anti-phosphotyrosine moAb, revealed a dramatic increase in fluorescence as compared to untreated control cells (fig 2).

To show that the effect of NEM is on the phosphorylation of the tyrosine residues a phosphoaminoacid analysis was performed. Almost a tenfold increase in the level of phosphotyrosine (0.3 to 2.8 %) in NEM-treated Jurkat T-cells was detected (fig 3).The level of phosphothreonine increased from 4.6 to 6 % (fig 3).

To test whether the NEM-induced phosphorylation on tyrosine is reversible a thiolreagent was used. When 2 mM DTT was added to NEM-treated Jurkat cells an accelerated dephosphorylation of the hyperphosphorylated substrates was seen in anti-P-Tyr immunoblots (fig 4). The hyperphosphorylation largely disappeared after 30 min of incubation at +37°C (fig 4).

The effect of NEM *in vivo* can either be on the TPKs or the PTPases. To address this question two types of experiments were performed. First, NEM had no effect on the kinase activity of membrane fractions of Jurkat cells assayed by ^{32}P-γ-ATP phosphorylation (fig 5). To the contrary orthovanadate and also PAO increased the phosphorylation (fig 5). This was probably caused by blocking of PTPase activity.

Second, to see whether NEM has any effect on PTPases *in vitro* , anti-CD45 immunoprecipitates from Jurkat cells were shortly pretreated with NEM, PAO or orthovanadate and the PTPase activity measured with 10 mM orthophospho-tyrosine as substrate. The result showed that 100 μM NEM blocked the PTPase activity of the immunoprecipitated CD45 (fig 6). The effect was comparable to that of PAO and orthovanadate (fig 6). In an additional experiment DTT was added to a NP-40 lysate (see methods) of NEM treated Jurkat cells. This treatment did not

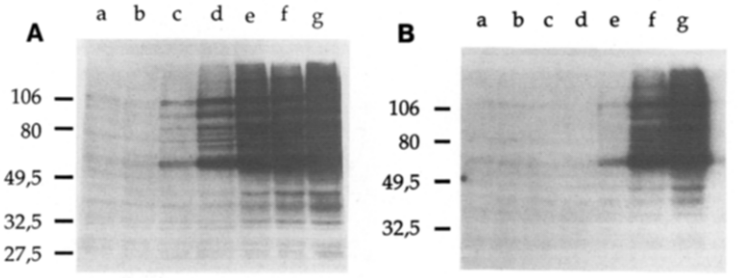

Figure 1. Panel A) Jurkat cells in culture medium were incubated at +37°C with 100μM NEM for b) 1, c) 3, d) 5, e) 7, f) 10, g) 20 minutes or a) no addition for 5 min. Panel B) Jurkat cells in culture medium were incubated for 20 min with b) 3, c) 10, d) 30, e) 50, f) 70 and g) 100 μM NEM or a) no addition. Incubations were ended through addition of boiling SDS sample buffer (v/v, 1:1).

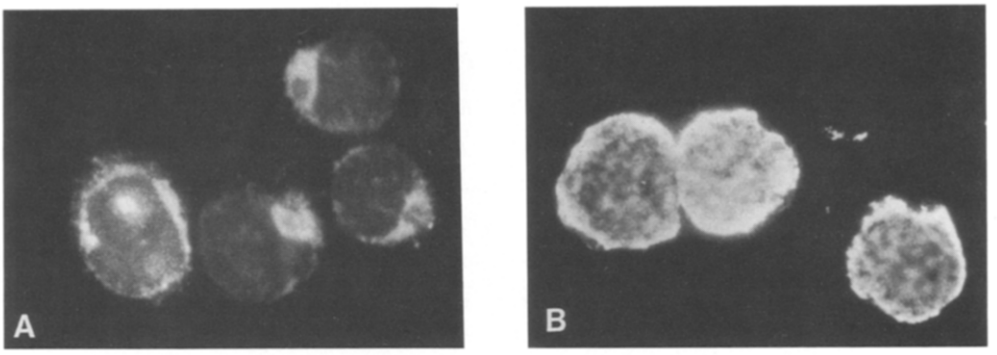

Figure 2. Cytocentrifuged Jurkat T-cells either A)untreated or B)NEM-treated (100μM, 20 min) were fixed in ice-cold methanol and stained with anti-phosphotyrosine moAb. FITC-conjugated sheep anti-mouse ab was used as the secondary ab.

affect the NEM-induced hyperphosphorylation (data not shown). Addition of NEM to a NP-40 lysates of untreated cells did not increase the phosphotyrosine content (data not shown).

Discussion

Data presented in this paper show that treatment of the T-cell line Jurkat with NEM at a concentration of 50 μM or higher within minutes induced a dramatic hyperphosphorylation on tyrosine residues. The phosphorylation peaked at 10-20 mins. Lower doses were also able to induce an increased tyrosine phosphorylation but required prolonged incubations (data not shown). It is possible that NEM as a hydrophobic molecule accumulates in the lipid bilayer membrane and therefore smaller doses also excert effects.

Sulfhydrylreagents have often been used in studies of glucose transport. Both NEM and PAO have been shown to inhibit the insulin activated hexose transport in adipose tissue cells (21, 22). And furthermore it has been shown that insulin stimulated tyrosine phosphorylation of a 15 kD

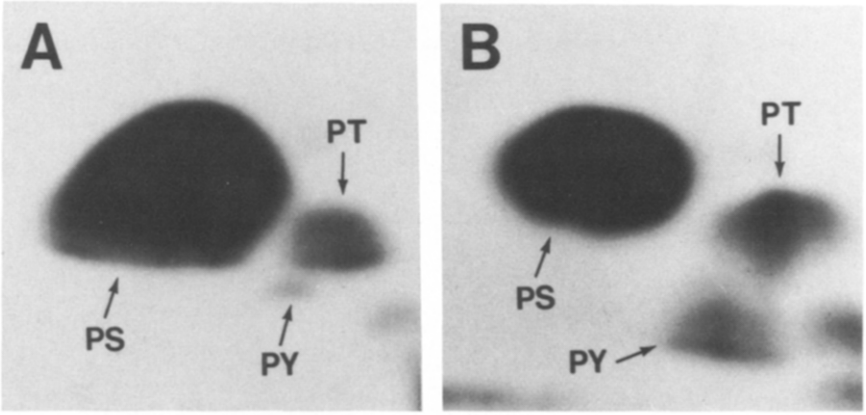

Figure 3. Jurkat T-cells labelled with ^{32}P-orthophosphate were A) untreated or B) treated with 100μM NEM for 20 min. Cells rapidly washed x3 with phosphate-free MEM were lysed with ice-cold 10% TCA. The phosphoaminoacid analysis was performed as described (see methods). Indicated are PY, phosphotyrosine, PT, phosphothreonine and PS, phosphoserine.

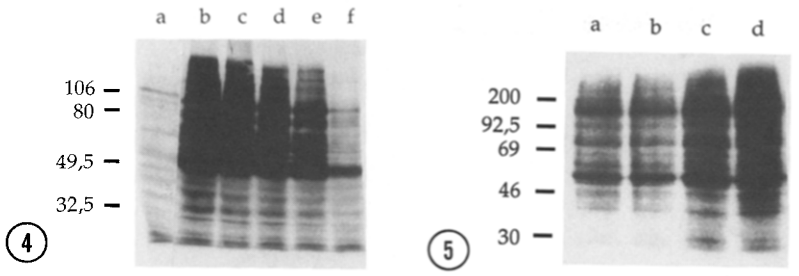

Figure 4. To Jurkat cells, treated with 100 µM NEM for 20 min, 2mM DTT was added and samples were further incubated at +37°C for b) 0, c) 1, d) 5, e) 15 and f) 30 min. Lane a) is untreated control. Incubations were ended through addition of boiling SDS sample buffer (v/v, 1:1).

Figure 5. Membrane fractions of Jurkat cells in kinase assay buffer (see methods) were incubated with for 5 min at +30°C with addition of lane b) 100 µM NEM, lane c) 100 µM PAO, lane d) 2 mM orthovanadate or lane a) no addition. Incubations were ended with boiling SDS sample buffer.

protein in 3T3-L1 adipocytes was augmented with PAO (23) while PAO did not affect insulin-triggered *in vitro* phosphorylation of cellular substrates in NIH 3T3 cells (24). These observations suggested an effect on PTPases, which was directly demonstrated in T-cells (25).

Tyrosine phosphatases have a conserved cysteine residue in their catalytic sites (10) This is susceptible to treatment with sulfhydrylreagents like PAO that has been shown to inhibit the CD45 PTPase (25). Therefore the effect of NEM on the PTPase activity in Jurkat cells was tested. In anti-CD45 immunoprecipitates from Jurkat cells both NEM and PAO inhibited the PTPase activity. But, NEM caused only a marginal inhibition of the PTPase activity in experiments with isolated Jurkat membranes, suggesting that other phosphatases in addition to the CD45 are of significance (data not shown).

Moreover, treatment with NEM did not alter the ^{32}P γ-ATP phosphorylation of substrates using isolated Jurkat membranes (fig 6). Altogether these results indicate that NEM and PAO distort the complex balance between phosphorylation and dephosphorylation by inhibiting the PTPase activity which leads to increased tyrosine phosphorylation.

As both NEM and PAO are lipidsoluble and strong sulfhydrylreagents they are also toxic to cells in prolonged assays. NEM offers anyhow a useful tool for studies on the phosphotyrosine dependent associations between various proteins or identification of elusive substrates for the TPKs.

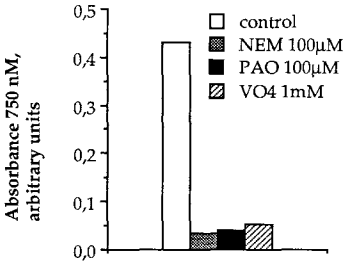

Figure 6. Jurkat cells were lysed with an ice-cold imidazole buffer and immunoprecipitation was performed with an anti-CD45 moAb. Washed immmunoprecipitates were incubated in a assay buffer for 30 min at +37°C with: 100 µM NEM, 100 µM PAO, 1 mM orthovanadate or without addition (as indicated). The level of absorbance of a control sample not incubated at +37°C was used as the reference level.

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