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TYROSINE PHOSPHORYLATION OF SHC IS INDUCED BY IL-3, IL-5 AND GM-CSF

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Summary: The receptors for IL-3, IL-5 and GM-CSF belong to the hematopoetic receptor superfamily and have no intrinsic tyrosine kinase activity but nevertheless indirectly induce protein tyrosine phosphorylation. In order to directly compare the effects of IL-3, IL-5 and GM-CSF on protein tyrosine phosphorylation we analyzed the murine cell line FDCP-1 which proliferates equally well in response to IL-3 and GM-CSF and the cell line B13 which responds to both IL-3 and IL-5. The protein tyrosine phosphorylation pattern induced by IL-3, IL-5 and GM-CSF in these cell lines was shown to be remarkably similar and all three cytokines induced tyrosine phosphorylation of Shc, a src homology domain-2 containing protein which has been shown to be involved in Ras activation by tyrosine kinase receptors.

The receptors for IL-3, IL-5 and GM-CSF are comprised of a common ß-chain and cytokine specific α-chains. The α-chains alone bind the respective cytokine with low affinity but are insufficient to transduce signal in the absence of the ß-chain. The ß-chain by itself does not appear to bind any of the three cytokines but is involved in the generation of high-affinity binding and in signal transduction (1). The common role of the ß-chain in signal transduction is in agreement with the redundancy that was found in the analysis of intracellular signalling events following stimulation of receptors by either of the three cytokines. Tyrosine phosphorylation of intracellular proteins was described to be similar after stimulation of factor-dependent cell lines with IL-3 and GM-CSF (2, 3, 4) or IL-3 and IL-5 (5). Moreover all three cytokines have been shown to activate Ras (6, 7).

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Abbreviations: IL, interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor.

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Several ways of Ras activation by membrane spanning receptors have been proposed (8). Shc, a src homology domain-2 containing protein has been described to be involved in Ras activation by tyrosine kinase receptors (9, 10, 11). Tyrosine phosphorylation of Shc after receptor stimulation leads to its association with the adaptor protein Grb-2 (11) which binds to the Ras activator Sos (12). In this report we addressed the question whether ligand stimulation of IL-3, IL-5 or GM-CSF receptors which do not belong to the family of tyrosine kinase receptors induce tyrosine phosphorylation of Shc.

Material and Methods

Cell lines and culture: B13, a B cell line (13) and FDCP-1, a myeloid cell line (14) were maintained in RPMI 1640 containing 10 % FCS, 50 µM 2-ME and 10% TS/A-IL5 (15) supernatant as a source of IL-5 (B13) or 10 % WEHI-3 (ATCC, Rockville, MD) supernatant as a source of IL-3 (FDCP-1).

Cell proliferation assay: Cells were cultured at a density of 5 x 10^3/200 µl in 96-well-flat-bottom microtiter plates with various concentrations of recombinant murine IL-3 (Genzyme), IL-5 (Genzyme) or GM-CSF (Genzyme) in RPMI 1640 containing 10% FCS and 50 µM 2-ME for 48 h. During the last 4 h of culture cells were pulse labelled with 0,5 µCi [^{3}H]thymidine (5 Ci/mmol sp. act.) and thymidine incorporation was quantified by scintillation counting as described (16).

Western blot analysis: Cells were factor-starved for 12-16 h in RPMI supplemented with 10 % FCS and 50 µM 2-ME. Stimulation was done at a concentration of 5 x 10^7cells/ml with an excess of recombinant murine cytokines (IL-3: 1000 U/ml, IL-5: 500 U/ml or GM-CSF: 40 U/ml) and was stopped after various periods of time with 8 volumes of PI/PBS (Phosphate Buffered saline containing 0,4 mM EDTA and 0,4 mM $NaVO_4$). Cells were pelleted, solubilized with lysis buffer (20 mM Tris.Cl, pH 8, 138 mM NaCl, 10% Glycerol, 1% NP-40, 0,025 mM p-nitrophenyl guanidinobenzoate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM $NaVO_4$, 2 mM EDTA, 10 mM NaF) and incubated on ice for 20 min. Cell debris were removed by centrifugation of the lysates at 14000 g for 15 min at 4°C. The cell lysates (1 x 10^6 cells/30 µl) were mixed with one volume of Laemmli buffer, boiled for 5 min and resolved by 7,5% SDS-PAGE under reducing conditions. The separated proteins were electrotranfered onto ECL-Hybond (Amersham) membranes in transfer buffer (25 mM Tris, 192 mM Glycine, 20 % Methanol, pH 8,3). Membranes were blocked in TBST-BSA (0,01 M Tris, pH 7,5, 0,1 M NaCl, 0,1 % Twen 20, 1% BSA) and incubated with horseradish peroxidase conjugated anti-phosphotyrosine mAb RC20 (Transduction Laboratories, Lexington, Kentucky) at 0,1 µg/ml TBST-BSA. Blocking (20 min) and antibody incubation (20 min) were done at 37°C. After washing in TBST membranes were treated with enhanced chemiluminescence developing solution (Amersham) according to the manufacturers instructions and exposed to ECL-Hyperfilms (Amersham).

Immunoprecipitation: Stimulation and solubilization of the cells were done as described above except that the cells were lysed at

a concentration of 5 x 10^6 cells/500 µl lysis buffer. Cell lysates were precleared with protein A sepharose beads (Pharmacia) conjugated with rabbit-anti-mouse-IgG (Cappel) for 1 h at 4°C. The precleared supernatants were incubated at 4°C with anti-Shc rabbit serum (10 µg/ml, UBI) for 1 h and subsequently with 20 µl protein A agarose (Santa Cruz) for 1 h. The immunoprecipitates were washed four times with lysis buffer, solubilized with Laemmli buffer and resolved by 7,5 % SDS-PAGE under reducing conditions.

Results and Discussion

The proliferative response of B13 cells to IL-3 or IL-5 and of FDCP-1 cells to IL-3 or GM-CSF was analysed as shown in Fig. 1. The dose response curve of B13 in the presence of IL-3 or IL-5 is very similar and the maximal proliferation induced by IL-3 or IL-5 is in the same range (Fig. 1a). Similar results were obtained for the IL-3 or GM-CSF induced proliferation of FDCP-1 cells (Fig. 1b). A plateau of the dose response was reached for B13 cells at 20 U/ml of IL-5 or 50 U/ml of IL-3, for FDCP-1 cells at 3 U/ml of GM-CSF or 500 U/ml of IL-3.

The cytokine induced protein tyrosine phosphorylation in FDCP-1 and B13 is shown in Fig. 2. Factor starved FDCP-1 cells were stimulated with an excess of IL-3 or GM-CSF for 5, 10 and 20 min, cell lysates were prepared and analysed by immunoblotting with an

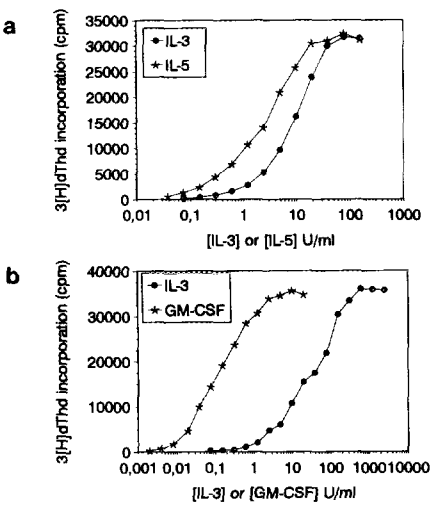
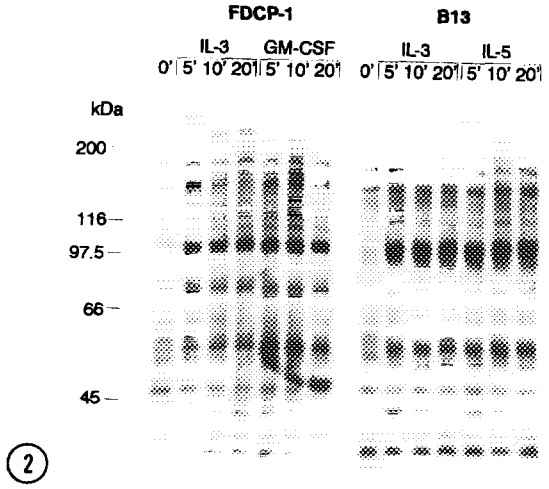
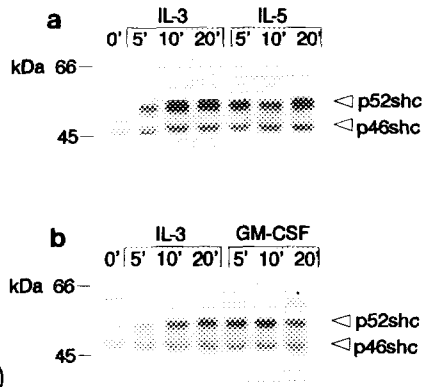


Fig.1. **Proliferation of B13 (a) and FDCP-1 (b) cells in response to cytokines.** [^{3}H] thymidine incorporation as an indicator of cellular proliferation is plotted against rising cytokine activities [U/ml]. The units of IL-3, IL-5 and GM-CSF are defined according to the manufacturer and therefore not comparable. This figure represents one of three independent experiments with similar results.



(2)



(3)

Fig. 2. **Tyrosine phosphorylation in B13 and FDCP-1 cells after cytokine stimulation.** Factor starved B13 and FDCP-1 cells were stimulated for the indicated time periods with IL-3 or IL-5 and IL-3 and GM-CSF, respectively. Lysates were prepared and immunoblotted with an anti-phosphotyrosine antibody. The observed tyrosine phosphorylation pattern was reproduced in two independent experiments.

Fig. 3. **Tyrosine phosphorylation of Shc is induced by IL-3, IL-5 and GM-CSF stimulation.** Factor starved B13 (a) and FDCP-1 (b) cells were stimulated for the indicated time periods with IL-3 or IL-5 and IL-3 or GM-CSF, respectively. Lysates were prepared and immunoprecipitated with anti-Shc rabbit serum. Precipitated proteins were immunoblotted with an anti-phosphotyrosine antibody. Identical results were obtained in multiple independent experiments. A control rabbit serum did not precipitate any tyrosine phosphorylated protein.

anti-phosphotyrosine antibody. The pattern of protein tyrosine phosphorylation induced by IL-3 and GM-CSF was identical. Both cytokines induced phosphorylation of proteins of 170, 145, 135, 125, 115, 108, 98, 78, 76 and 56 kDa. GM-CSF induced a more profound phosphorylation of the 145, 135, 125, 115, 108 and 58 kDa proteins after 5 and 10 min which declined after 20 min to a similar level as the IL-3 induced phosphorylation. Stimulation of factor-starved B13 cells with IL-3 or IL-5 for 5, 10 and 20 min and subsequent analysis of tyrosine phosphorylation likewise revealed an identical pattern of protein phosphorylation for both cytokines which moreover seemed to be similar to the pattern induced by IL-3 or GM-CSF in FDCP-1 cells. However, the degree of tyrosine phosphorylation of proteins with similar molecular mass varied in the different cell lines. For example, a 76 kDa protein was only weakly phosphorylated in B13 cells after cytokine stimulation whereas more pronounced phosphorylation of a 76 kDa

protein was found in cytokine stimulated FDCP-1 cells. Furthermore, a 40 kDa protein was found to become tyrosine phosphorylated in B13 cells only.

Our data are in agreement with observations by others showing overlapping tyrosine phosporylation patterns for IL-3 and GM-CSF in MO7E (2), DA-3 (3) and AML-193 (4) cells and for IL-3 and IL-5 in T88-M cells (5). Interestingly, tyrosine phosphorylation patterns of FDCP-1 and B13 cells appear rather similar although B13 derives from the B-cell lineage and FDCP-1 is of myeloid origin, suggesting conserved signalling events in cells of different differentiation. Considering previous and the current studies it is reasonable to accept as a working hypothesis that the intracellular signals delivered by the receptors of the three cytokines are identical. Thus the specifity of the biological effects may be solely determined by the differential expression of the α-chains on certain cell lineages and the local presence of the different cytokines. However, it would be premature to exclude that the α-chains themselves may contribute a cytokine specific signal. At least it has been shown that the cytoplamic domain of the IL-5 receptor α-chain is essential for signal transduction because in contrast to the wildtype chain a truncated IL-5 receptor α-chain lacking the cytoplasmic domain fails to stimulate IL-5 driven proliferation after transfection in FDCP-1 cells (17).

Because each of the cytokines has been described to activate Ras we addressed the question if Shc, a protein implicated in Ras activation, becomes tyrosine phosphorylated after stimulation with IL-3, IL-5 or GM-CSF. Shc has been shown to exist in three isoforms: a 52 and a 46 kDa protein which are ubiqitously expressed and a 66 kDa proteins which seems to be differentially expressed (9). The anti-Shc rabbit serum which was used for immunoprecipitation recognizes all three isoforms. Factor-starved B13 and FDCP-1 cells were stimulated with their respective cytokines for 5, 10 and 20 min and immunoprecipitated with anti-Shc serum. As shown in Fig. 3b, IL-3 as well GM-CSF induced strong tyrosine phosphorylation of $p52^{shc}$ and somewhat weaker phosphorylation of $p46^{shc}$ in FDCP-1 cells. Similarly, IL-3 and IL-5 induced tyrosine phosphorylation of $p52^{shc}$ and $p46^{shc}$ in B13 cells (Fig. 3a). In both cell lines IL-3 induced Shc phosphorylation appears to achieve his maximal level after 10 min in contrast to IL-5 and GM-CSF which already stimulate maximal phosphorylation after 5 min.

Our results show that all three cytokines stimulate tyrosine phosphorylation of $p52^{shc}$ and $p46^{shc}$. This indicates that the receptors for IL-3, IL-5 and GM-CSF, which have no intrinsic tyrosine kinase activity apparently use a similar intracellular pathway for Ras activation as tyrosine kinase receptors. A similar observation has been recently made for the IL-2 receptor. We and others have shown that IL-2 receptor stimulation induces tyrosine phosphorylation of $p52^{shc}$ in T- and B-cell lines (18, 19). The finding that IL-2 in contrast to IL-3, IL-5 and GM-CSF only stimulates tyrosine phosphorylation of $p52^{shc}$ and not $p46^{shc}$ although both proteins are expressed in the respective cell line raises the question whether functional differences for the Shc isoforms may exist.

While this work was in progress it has also been shown that the erythropoietin and the IL-3 receptor stimulate tyrosine phosphorylation of $p52^{shc}$ (20). Like the receptors for IL-3, IL-5 and GM-CSF the IL-2 receptor ß-chain and the erythropoietin receptor belong to the hematopoetic receptor superfamily (21). However, not all receptors of this family induce tyrosine phosphorylation of Shc because we have recently shown that the IL-7 does not stimulate Shc tyrosine phosphorylation in a pre-B cell line (18). In this regard it is of interest that very recent reports demonstrated the existence of a common chain, the γ_c-chain, for the IL-2, IL-4 and IL-7 receptors (22-24). The role of the common γ_c-chain seems to differ from the role of the common $ß_c$ -chain in the IL-3/IL-5 and GM-CSF receptor system, because IL-2, IL-4 and IL-7 have been described to induce different intracellular signals (6, 7, 18, 25) and their biological activities are less redundant than those of IL-3, IL-5 and GM-CSF.

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