Interleukin-4 Induces Activation of Mitogen-activated Protein Kinase and Phosphorylation of Shc in Human Keratinocytes

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Most cytokines stimulate the p21ras pathway, leading to MAP kinase activation. One exception is interleukin-4 (IL-4), which has been shown not to activate this pathway in hematopoietic cells. However, IL-4 acts on a broad range of cells, including keratinocytes, in which it induces IL-6 production. We report here that IL-4 stimulation of human keratinocytic cell lines or primary cultures activates MAP kinase. In these cells, IL-4 stimulation induces the tyrosine phosphorylation of p42/44 MAP kinase as well as its catalytic activity. We also observed an increased phosphorylation of p46Shc, an SH2-containing protein involved in the Ras pathway, as a result of IL-4 stimulation in human keratinocytic cell lines but not in T lymphocytes.

IL-4 is a cytokine produced by cells of hematopoietic origin, which acts on a broad range of cells of both hematopoietic and non-hematopoietic origin (1, 2). Among the latter, epidermal keratinocytes, which represent the major cell type of the skin, are able to produce IL-6 in response to IL-4 stimulation (3). Even though the IL-4 receptor (IL-4R) lacks a tyrosine kinase domain, IL-4 stimulation results in increased tyrosine phosphorylation of a variety of intracellular substrates, including a 170-kDa protein, designated as 4PS in hematopoietic cells (4–6). 4PS is both structurally and functionally similar to IRS-1, the most prominent substrate phosphorylated in response to insulin or insulin-like growth factor-1 stimulation. 4PS is one of the first substrates phosphorylated in response to insulin or insulin-like growth factor-1 stimulation in cells of connective tissue origin (7, 8). The amino acid sequence of 4PS (now called IRS-2) has recently been reported, and its alignment with that of IRS-1 revealed highly conserved stretches, which encompass the domains of interaction with SH2-containing proteins p85 and Grb2 (8). IL-4 induces the phosphorylation of 4PS/IRS-1 but fails to activate the p21ras pathway in hematopoietic cells. Indeed, neither phosphorylation of Shc nor the conversion of Ras to its active GTP-bound conformation has been observed, correlating with a lack of Raf-1 or MAP kinase activation (9, 10). However, IL-4 does activate the phosphatidylinositol 3-kinase whose p85 subunit is known to bind to 4PS in an IL-4-inducible manner (4, 11).

IL-4 down-regulates IL-6 production in human and murine monocytes, in contrast to what is described for keratinocytes (3, 12). Thus, IL-4 regulates the IL-6 gene in opposite ways depending on the origin of its target cells (hematopoietic or not). We therefore investigated the possibility that alternate signal transduction pathways, which could account for these differences, may be triggered in various cell types. We indeed observed an increased tyrosine phosphorylation of MAP kinase, which is accompanied by an enhanced catalytic activity of the enzyme following IL-4 stimulation. We also report here that in keratinocytes, but not in T cell blasts, Shc is in fact phosphorylated, suggesting its possible implication in MAP kinase activation in keratinocytes in response to IL-4.

EXPERIMENTAL PROCEDURES

Materials and Antibodies

Recombinant human IL-4 was kindly provided by DNAX Corp. (Palo Alto, CA). MAP kinase substrate, anti-Tyr(P) (4G10), and anti-Shc were from Upstate Biotechnology Inc. Anti-MAP kinase antibody (sc 154) was from Santa Cruz Biotechnology, Inc.

Cell Culture

SVK14 (13) and A431 keratinocytic cell lines were seeded at 5 × 104 cells/100-mm Petri dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 3 days. Primary human keratinocytes (PHK) were isolated from human foreskin of a 1-year-old donor as described by Noël-Hudson et al. (14) and were grown in keratinocyte serum-free medium supplemented with EGF (1 ng/ml) and bovine pituitary extracts. Cells were seeded at 105 cells/100-mm Petri dish and grown for 8 days. Cells were starved by incubation with 0.1% fetal calf serum for SVK14 cells (48 h) and for PHK (24 h) or without serum for A431 cells (48 h), before stimulation with IL-4 (50 ng/ml) for increasing periods of time.

Peripheral blood lymphocytes were obtained from lymphocyte concentrates of healthy donors by Ficoll. Cells (109/ml) were prestimulated with 1 μg/ml purified phytohemagglutinin (Wellcome, France) for 5 days. Cells were further expanded with IL-2 (7.5 ng/ml) for 5–7 days before use. These cells are IL-2-dependent for growth and were rendered quiescent by IL-2 deprivation for 24 h before stimulation with IL-4 (50 ng/ml).

Immunoblotting and Immunoprecipitation

Cell-free lysates were prepared as described previously (15). For immunoprecipitation 500 μg of protein were incubated with the required quantities of antibodies. Immune complexes were recovered on protein A-Sepharose (15).

MAP Kinase Assays

Phosphorylation of Exogenous Substrate—Cell-free extracts were prepared in buffer containing 50 mM β-glyceroephosphate, 100 μM Na2VO4, 2 mM MgCl2, 1 mM EGTA, 0.5% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM DTT, and the determination of MAP kinase activity was performed as described previously (16).

In-gel Kinase Assays—Cellular extracts were prepared as for immunoblotting. Kinase assays in SDS-PAGE were carried out by using the procedure of Cano et al. (17). Briefly, polyacrylamide (14%) minigels were cast with 0.5 mg of MBP/ml added to the SDS-polyacrylamide gel solution prior to polymerization. After electrophoresis, SDS was removed by incubation for 1 h in 250 ml of 20% isopropyl alcohol in 50 mM Tris-HCl (pH 8.0) followed by 1 ml DTT, 50 mM Tris-HCl (pH 8.0). To
factors involved in IL-6 promoter regulation (18), we investi-
gated the possibility of MAP kinase by IL-4 in A431 and SVK14 cells. As shown in Fig. 1, a time-dependent stimu-
lation of MAP kinase, as measured by peptide substrate phos-
phorylation, was observed in both cell lines following treatment
with IL-4. The time course of stimulation appeared to be
slightly different in the two cell lines; maximal activation
was observed after 10 min of stimulation in A431 cells, whereas it
peaked at 2 min after stimulation of SVK14 cells. In addition,
the intensity of the response was higher in SVK14 cells. The
property of IL-4 to stimulate MAP kinase activity was also
analyzed in PHK; the kinetics of MAP kinase activation in
PHK was superimposable to that in A431 cells and PHK (max-
imal activity occurring at 10 min). At this time, a 2.5-fold
enhancement of MAP kinase activity was observed (Fig. 2). In
SVK14 cells, the time course of MAP kinase activation after
EGF stimulation was similar to that observed with IL-4,
whereas with A431 and PHK cells it was slower and lower (not
shown and Fig. 2, respectively), possibly due to an attenuation
of the MAP kinase cascade stimulated by other growth factors
(19, 20). Indeed, A431 cells are known to overexpress EGF
receptors and to produce transforming growth factor α or am-
phiregulin, which are able to interact with and constitutively
stimulate EGF receptors, implicating a constitutive activation
of MAP kinase cascade (21). In agreement with other reports
(9, 10), no modification of MAP kinase catalytic activity was
observed in cellular extracts from T cell blasts after IL-4 stim-
ulation (not shown).

Stimulation of MAP kinase activity was also analyzed using
an in-gel kinase assay, with immobilized MBP as substrate.
Following IL-4 stimulation, two bands of Mr 42,000 appeared to
be phosphorylated reflecting the catalytic activity of
kinases of the corresponding Mr (Fig. 1B, inset). Quantiﬁca-
tion of 32P incorporated in the two bands indicated that —2-fold
activation of MAP kinase activity was observed in SVK14 cells
at 5 min, whereas a 1.7-fold enhancement was observed in
A431 cells at 20 min (Fig. 1B). MAP kinase was therefore
activated in the two keratinocytic cell lines. Since in SVK14
cells, the time course and intensity of the response were similar
to those obtained with other growth factors, these cells were
chosen for further investigations.

IL-4 Stimulates the Tyrosine Phosphorylation of MAP Kinase
in Human Keratinocytic Cell Lines but Not in T Cell Blasts—We
then examined the level of tyrosine phosphorylation
of MAP kinase in SVK14 cells in comparison with T cell
blasts after IL-4 stimulation. A time-dependent tyrosine phos-
phorylation was observed in a protein of Mr, 42,000 (Fig. 3A).
Stripping the blot and reprobing with an anti-MAP kinase

RESULTS AND DISCUSSION

MAP Kinase Is Activated by Stimulation of IL-4 Receptor in
Human Keratinocytes—Preliminary experiments indicated
that cells from the human keratinocytic cell lines A431 and
SVK14 express IL-4 receptors and produce IL-6 in response to
IL-4 stimulation. Since the Ras/MAP kinase pathway is known
to lead to phosphorylation of NF-IL-6, one of the transcription
factors involved in IL-6 promoter regulation (18), we investi-
gated the level of tyrosine phosphorylation of MAP kinase in
SVK14 cells after stimulation with IL-4 (Fig. 3B). MAP kinase
activity was determined as in Fig. 1A. The average of three
independent experiments is shown.

**Fig. 1. MAP kinase activity after IL-4 stimulation.** A, phosphor-
ylation of exogenous substrate. Cell lines were stimulated for increasing
periods of time and lysed. MAP kinase activity was determined by
incubating the lysate of SVK14 cells (●) or A431 cells (○) with MAP
kinase substrate peptide and [γ-32P]ATP. The average of six independent
experiments is shown, and MAP kinase activity is represented as
-fold activation of the level observed in unstimulated cells. B, in-gel
kinase assay. Lysates from untreated or IL-4-stimulated A431 or
SVK14 cells were used to determine MAP kinase activity in an in-gel
kinase assay of renatured proteins in PAGE in the presence of MBP as
substrate. Inset: 32P incorporated into the p42/p44 bands. The graph
indicates quantification of radioactivity incorporated in the two bands.

Affinity Purification of Shc with Immobilized Grb2

Recombinant human Grb2 protein was coupled to activated CNBr-
Sepharose 4B as recommended by the supplier (Pharmacia Biotech Inc.).
Cellular extracts (500 µg) prepared as for immunoblotting were
then incubated with immobilized Grb2 overnight at 4°C. After several
washings in immunoprecipitation buffer, beads were resuspended in
SDS sample buffer, heated at 100°C for 5 min, and immunoblotted as
indicated.
antibody showed that the level of MAP kinase immunoprecipitated with anti-Tyr(P) (P-Tyr, A) or anti-MAP kinase (MAP K, B) antibody increased with time and followed the kinetics of phosphorylation, beginning at 2 min and detectable for at least 10 min. Experiments performed under the same conditions with cellular extracts of T cell blasts showed no evidence for MAP kinase tyrosine phosphorylation (Fig. 3A).

Anti-MAP kinase immunoprecipitation followed by an anti-Tyr(P) immunoblotting in cellular extracts from SVK14 cells (Fig. 3B) also showed that MAP kinase is tyrosine-phosphorylated in keratinocytes but not in T cell blasts (not shown). These results suggested that specific signaling pathways might occur in keratinocytes.

IL-4 Stimulates the Phosphorylation of Shc in Human Keratinocytes and T Cells—It has been previously described that in addition to Grb2/SOS, another adaptor, the Shc protein, binds simultaneously to growth factor receptors and to Grb2 in a phosphotyrosine-dependent manner and mediates signals from many tyrosine kinases (22). Immobilized Grb2 was used to affinity purify Shc from cellular extracts of SVK14 cells and T cell blasts and investigate its phosphorylation in response to IL-4. Shc exists in three isoforms of 46, 52, and 66 kDa. As shown in Fig. 4, in SVK14 cells, all three Shc proteins are able to associate with Grb2 in vitro in the absence of stimulation, whereas in T cell blasts only p46Shc and p52Shc are detectable, consistent with the absence of p66Shc in most hematopoietic cells (23). This stimulation-independent ability of Shc to bind Grb2 is presumably the result of interactions between the glycine/proline-rich region of Shc and Grb2 SH3 domain (22). No modification of the level of Shc proteins recovered on Grb2 beads was observed in T cell blasts after IL-4 stimulation. In contrast, in SVK14 cells, the level of the p46Shc protein increased with stimulation, whereas the level of the other associated Shc proteins remained unchanged. This inducible association of Shc to Grb2 is likely to reflect Grb2-SH2 binding to phosphorylated Shc. Indeed, immunoblotting with anti-Tyr(P) antibody showed that p46Shc became tyrosine-phosphorylated after 5 min of IL-4 stimulation in SVK14 cells (Fig. 4). This was also true for A431 and PHK cells (not shown), whereas no tyrosine phosphorylation of Shc was observed in T cell blasts (Fig. 4).

Our results suggest that Shc phosphorylation may be the critical step leading to MAP kinase activation by IL-4 in keratinocytes. Indeed, using insulin receptor-transfected hematopoietic 32D cells, which are defective in 4PS/IRS-1 and cannot therefore use the IRS-1/Grb2 pathway, it has been shown that stimulation by insulin led to concomitant activation of MAP kinase and phosphorylation of Shc (24). In addition, it has been shown that insulin or IL-4 induced similar levels of IRS-1 phosphorylation in an IL-4R-transfected, insulin-responsive, L6 myoblast cell line. Yet only insulin induces tyrosine phosphorylation of Shc and its association with Grb2, correlating with MAP kinase activation, and it has been suggested that this could explain the lack of MAP kinase activation by IL-4 (25). In addition Shc phosphorylation is induced by many cytokines, which activate the Ras/MAP kinase pathway (26–28), suggesting that Shc could play a critical role in linking cytokine receptors to ras activation. The data presented support the hypothesis that in keratinocytes the phosphorylation of Shc may be the link between IL-4R and MAP kinase. It has been reported also that insulin can significantly stimulate phosphatidylinositol 3-kinase and MAP kinase activities, although to a lesser extent in IRS-1-deficient mice, reinforcing the idea that MAP kinase stimulation may be driven by IRS-1-independent signaling pathways (29). These pathways could be used by IL-4R to activate the MAP kinase cascade.

Novel phosphotyrosine binding (PTB) domains have been described (30). Insulin-stimulated phosphorylation of IRS-1 and Shc is mediated by interaction of these two proteins via their PTB domain (31, 32) with a NPXY domain in the juxtamembrane region of insulin receptor β subunit. Although a similar NPXY motif is also present in IL-4R and is required for IRS-1 phosphorylation (33), no tyrosine phosphorylation of Shc after IL-4R stimulation has been reported (34), suggesting that this sequence is not sufficient to interact efficiently with Shc. It has been suggested that amino acids surrounding the NPXY motif may be critical in the regulation of interactions between proteins that possess PTB motif (35). To bypass the lack of interaction, Shc can also be phosphorylated on tyrosine by certain members of the Src tyrosine kinase family (36). The distribution of members of this family is different between lymphocytes and keratinocytes, and implication of different Src kinases could explain the difference of Shc phosphorylation observed between these two cell types. Further experiments are currently in progress to identify which members of the Src kinase family are involved in this phosphorylation of Shc.

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