We have characterized and analyzed IGF-I- and insulin-stimulated cell growth, receptor binding, and autophosphorylation in the human leukemic cell line HL-60. IGF-I-stimulated cell growth occurred at low (5 ng/ml) and insulin stimulated only at high (500 ng/ml) concentrations. Binding of 125I-IGF-I to partially purified plasma membrane proteins followed the characteristics of IGF-I receptor binding. 125I-IGF-I binding, as determined by chemical cross-linking, occurred to a 145-kDa protein. IGF-I, as well as insulin, stimulated the autophosphorylation of a 105-kDa band (pp105), but we could not detect a 95-kDa band corresponding to the known molecular mass of the IGF-I receptor subunits. Phosphorylation of pp105 followed the dose-response characteristics of the IGF-I receptor. The phosphorylation of pp105 occurred at tyrosine and threonine, and the pattern of HPLC tryptic peptide maps showed marked differences when compared with that of a phosphorylated insulin receptor β-subunit. Enzymatic deglycosylation of pp105 resulted only in a slight reduction of the molecular weight.

These data suggest that pp105 is the β-subunit of an IGF-I receptor variant with a higher molecular weight, similar to that found in fetal tissue. The IIL-60 cell may acquire, at least in part, malignant growth characteristics through reexpression of the fetal version of the IGF-I receptor.

Leukemic cells possess a growth advantage over their normal counterparts. Although there are indications that polypeptide growth factors and their membrane receptors may play an important role, it is not known which molecular mechanisms are responsible for this growth advantage. In theory, autonomous production of growth factors (autocrine hypothesis) or increased susceptibility to physiological growth-promoting agents (altered signal transduction hypothesis) could be responsible for their altered growth behavior. HL-60 cells (1) are highly dependent upon the presence of transferrin and insulin under serum-free culture conditions (2). Since supraphysiological concentrations of insulin (500 ng/ml) are required it is likely that this effect occurs through the IGF-I receptor and that IGF-I is the physiological stimulus for this growth-promoting effect.

Materials—Porcine insulin was purchased from Novo Industries (Denmark), recombinant human IGF-I and IGF-II were gifts from Chiron Co., San Francisco, CA and Eli Lilly Co., respectively. 1,25P3 ATP (2900 Ci/mmol) was from New England Nuclear (Federal Republic of Germany), and [3H]iodotyrosyl IGF-I was from Amersham International, United Kingdom. Disuccinimidyl suberate and glycopeptidase P were from Boehringer Mannheim and neuraminidase from Behring, Marburg, FRG. Aprotinin, phosphomethylphenyl fluoride, leupeptin, and pepstatin were from Sigma (FRG), and wheat germ agglutinin coupled to agarose was from Miles (Israel). Triton X 100 and all reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Bio-Rad. All other reagents were of the best grade commercially available.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin was purchased from Novo Industries (Denmark), recombinant human IGF-I and IGF-II were gifts from Chiron Co., San Francisco, CA and Eli Lilly Co., respectively. [3H]ATP (2900 Ci/mmol) was from New England Nuclear (Federal Republic of Germany), and [3H]iodotyrosyl IGF-I was from Amersham International, United Kingdom. Disuccinimidyl suberate and glycopeptidase P were from Boehringer Mannheim and neuraminidase from Behring, Marburg, FRG. Aprotinin, phosphomethylphenyl fluoride, leupeptin, and pepstatin were from Sigma (FRG), and wheat germ agglutinin coupled to agarose was from Miles (Israel). Triton X 100 and all reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Bio-Rad. All other reagents were of the best grade commercially available.

HL-69 Cell Culture—HL-60 cells (9.5 × 10^6/ml) were kept in continuous suspension culture in Iscove's modified Dulbecco's medium (Gibco) supplemented with 5% fetal calf serum (Gibco) in roller bottles (Falcon) at 37 °C, 5% CO2, and harvested at a final concentration of 1.5–2 × 10^6/ml. For quantitative proliferation assays 0.25 × 10^6 cells were seeded in a chemically defined medium (Iscove's modified Dulbecco's medium) containing a constant amount of transferrin (5 μg/ml) and various concentrations of IGF-I or insulin. Viable cells (trypan blue exclusion) were counted under the microscope.
Receptor Preparation—1.5-2 x 10^9 HL-60 cells were centrifuged at 900 rpm, and the pellet was washed 3 times with Krebs-Ringer-Hepes buffer (pH 7.4). Cells were lysed by freezing and thawing 3 times in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (6 mmol/liter), aprotonin (1200 trypsin inhibiting units (TU/liter), leupeptin (2 µmol/liter), pepstatin (2 µmol/liter), benzamidine (10 mM), and phenylmethylsulfonyl fluoride (2 mM/liter) in a buffer containing NaH_2PO_4 (pH 7.4, 10 mM/liter), EDTA (5 mM/liter), and sucrose (250 mM/liter). Subsequently, the cell lysate was centrifuged for 50 min at 200,000 x g at 4 °C. The supernatant was discarded, the pellet was dissolved in 25 mM/liter Hepes buffer, 1% Triton X-100, aprotonin (600 TU/liter), leupeptin (2 µmol/liter), pepstatin (2 µmol/liter), and phenylmethylsulfonyl fluoride (2 mM/liter) and centrifuged for 50 min at 200,000 x g to remove insoluble material. The supernatant was then applied to a column of wheat germ agglutinin coupled to agarose. After extensive washing with 25 mM/liter Hepes buffer, pH 7.4, containing 0.1% Triton X-100, the bound material was eluted with 25 mM/liter Hepes buffer containing 0.1% Triton X-100 supplemented with 0.3 M/liter N-acetylglucosamine. Human muscle cells from gastrocnemius muscle of non-diabetic patients, which had been obtained during surgery, were freed of vessels and fat tissue. About 8-10 g of muscle was used for each receptor preparation [15].

Binding of ^125I-IGF-I Binding to WGA-Purified Receptor—Samples containing about 3 µg of solubilized and WGA-purified receptor protein were incubated with 20,000 cpm of ^125I-IGF-I and various concentrations of unlabeled IGF-I, IGF-II, and insulin for 45 min at 22 °C in a solution containing 50 mM/liter Tris-HCl, pH 7.5, 10 mM/liter NaF, 20 mM/liter sodium N-ethylnorvasate, 10 mM/liter Tris-HCl, and 1% bovine serum albumin. Separation of receptor and receptor-bound IGF-I was performed by using dextran-coated charcoal (30). The amount of ^125I-IGF-I bound to the receptor was determined in a γ-counter.

Affinity Cross-linking of ^125I-IGF-I to WGA-purified HL-60 Receptor—Cross-linking experiments were performed as described (16, 31). 40 µl (4 µg of protein) of WGA-purified receptor eluate was incubated with 4 mM MgSO_4, 0.2% bovine serum albumin, and 0.5 nM ^125I-IGF-I (46,000 cpm) in the presence or absence of 100 nM unlabeled peptide in a final volume of 80 µl for 1 h at 22 °C. Tubes were chilled on ice and 3 µl of dithiothreitol (7 mM in dimethyl sulfoxide) was added for 20 min at 4 °C. The incubation was terminated by addition of Laemmli buffer containing 160 mM dithiothreitol and subsequent boiling for 25 min at 95 °C. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (7.5% gel) and autoradiography.

Receptor Phosphorylation—Approximately 5 µg of WGA-purified receptor protein was preincubated at 22 °C for 30 min with insulin or IGF-I in different concentrations (0-1000 nM/liter), followed by an incubation with [γ-^32P]ATP (10 µCi/µl) in elution buffer containing 10 mM/liter MnCl_2 and 1 mM/liter vanadate at 22 °C for 10 min. The incubation was terminated by the addition of Laemmli buffer containing 100 mM/liter dithiothreitol and subsequent boiling for 25 min at 95 °C. The phosphopeptides were separated by 7.5% polyacrylamide gel electrophoresis, identified by autoradiography, cut out of the gel, and Cerenkov radiation was measured.

Immunoprecipitation—The partially purified receptor protein from HL-60 was phosphorylated as described above. Phosphorylation was stopped by addition of a Hepes buffer (25 mM/liter, pH 7.4) with 200 µmol/liter NaF, 90 µmol/liter sodium pyrophosphate, 10 mM/liter EDTA, 10 µmol/liter ATP, 0.4 µmol/liter vanadate, and 2400 TU/liter aprotinin. The phosphoproteins were then immunoprecipitated with anti-insulin receptor receptor serum.

Neuraminidase and Glycopeptidase F Digestion—10 µg of receptor protein was phosphorylated as described above. The 105-kDa phosphoprotein was purified from the incubation and stop buffer by a 10-fold dilution with deionized water and subsequent concentration of the phosphoprotein with a Centricon 10 column (Amicon) until the original volume was regained (180 µl). The procedure was repeated 3 times until a final 1000-fold dilution was achieved. The solution was then transferred to two Eppendorf cups (90 µl each) and lyophilized in a Speed Vac concentrator (Bachhofer). The proteolysis was terminated by the addition of Laemmli buffer containing 100 mM/liter dithiothreitol and subsequent boiling for 25 min at 95 °C. The phosphoprotein was separated by 105 kDa polyacrylamide gel electrophoresis (7.5% gel) and autoradiography.

RESULTS

The leukemic promyelocytic cell line HL-60 can be continuously grown in serum-free medium provided it contains insulin (2) or IGF-I (4), respectively, and transferrin. When we compared the growth stimulatory effects of various concentrations of IGF-I and insulin in the presence of a constant amount of transferrin we found IGF-I to be a more potent stimulator than insulin (Fig. 1).

To analyze IGF-I signal transmission by the target cell, glycoproteins from HL-60 membranes were partially purified with a WGA column. High affinity binding sites for IGF-I could then be demonstrated by displacement of ^125I-IGF-I with increasing concentrations of unlabeled IGF-I, IGF-II, and insulin. IGF-I competed in low concentrations, whereas displacement with IGF-II and insulin was less effective at the same concentrations. IGF-I, however, was more potent in displacement than insulin (Fig. 2).

To identify the membrane protein responsible for the high

![Fig. 1. Growth of HL-60 cells in suspension culture in the presence of IGF-I or insulin. Each point represents the mean value and standard deviation of three experiments.](http://www.jbc.org/3)
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Fig. 2. Competition curves of \( ^{125}\text{I}-\text{IGF-I} \) binding. The WGA-purified IGF-I receptor was incubated with \( ^{125}\text{I}-\text{IGF-I} \) and increasing concentrations of unlabeled peptide (IGF-I, IGF-II, insulin) for 45 min at 22 °C. Binding of \( ^{125}\text{I}-\text{IGF-I} \) is expressed as percentage of maximum binding obtained in the absence of unlabeled peptides. Each point represents the mean value of two separate experiments.

Fig. 3. Autoradiogram showing the reaction products of cross-linking of \( ^{125}\text{I}-\text{IGF-I} \) (0.5 nM/liter) to the WGA-purified HL-60 receptor with disuccinimidyl suberate (0.25 mM/liter). Experiments were performed in the absence (A) or presence of unlabeled IGF-I (B), IGF-II (C), and insulin (D). The concentration of unlabeled protein was for each experiment 100 nM/ml. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (7.5% gel) under reducing conditions (150 mM dithiothreitol).

When glycoproteins isolated from HL-60 membranes were phosphorylated and subsequently immunoprecipitated with an antibody raised against the insulin receptor, but also cross-reacting with the IGF-I receptor, separation of the immunoprecipitate on gel electrophoresis revealed an insulin-stimulated 105-kDa phosphoprotein (Fig. 4).

When we compared phosphoproteins from HL-60 cells to the insulin receptor \( \beta \)-subunit of human adult skeletal muscle under identical experimental conditions, a clear molecular mass difference of 10 kDa between the HL-60 protein and the insulin receptor \( \beta \)-subunit became evident (Fig. 5).

Tryptic digestion and subsequent HPLC peptide mapping

Fig. 4. Autoradiogram showing 105-kDa phosphoprotein. The WGA-purified protein from HL-60 was incubated in the absence (Ins–) and presence (Ins+) of 100 nM/liter insulin and phosphorylated with [γ-\( ^{32}\text{P} \)ATP for 10 min at 22 °C.

Fig. 5. Autoradiogram showing the immunoprecipitated phosphoprotein from HL-60 cells (HL-60) and human muscle (HM). Both HL-60 and human muscle cell phosphorylation were stimulated with 100 nM/liter insulin.
of pp105 isolated from HL-60 cells and of insulin receptor β-
subunit purified from human skeletal muscle showed different
characteristics (Fig. 6). Although phosphopeptide fragments
were eluted at the same acetonitrile concentrations, there
were marked differences in the phosphorylation pattern. In-
sulin receptor showed rather hydrophilic phosphopeptide
fragments with maximal 32P incorporation at 16.5% aceto-
nitrile (fraction 61) and 18.2% acetonitrile (fraction 70). In
contrast, the HL-60 derived 105-kDa phosphoprotein showed
only low 32P incorporation in these fractions and maximal
incorporation in a more hydrophobic phosphopeptide frag-
ment which eluted at 20.5% acetonitrile (fraction 83).

In order to characterize the phosphorylation sites of the
105-kDa phosphoprotein of HL-60, we analyzed the phospho-
amino acid content of the HL-60 HPLC fractions(Fig.7):
phosphotyrosine is predominant in pp105 from HL-60 (peaks
2–5) but, in addition, phosphothreonine is found (peak 6),
which corresponds to the fraction with the highest 32P incor-
poration (20.5% acetonitrile) of all the tryptic peptides.

When incubated with different IGF-I or insulin concen-
trations the phosphorylation of the pp105 occurred in a con-
centration-dependent manner. Fig. 8 shows the mean values of
dose-response curves of five individual receptor phosphoryl-
ation experiments after incubation with IGF-I or insulin,
respectively. Half-maximal 32P incorporation after stimu-
lation with IGF-I is found at a 100-fold lower concentration
(0.1 nM) than with insulin (10 nM). Maximal 32P incorpora-
tion for both ligands is present at concentrations between 100 and
1000 nM. This extreme sensitivity of pp105 phosphorylation
to IGF-I strongly suggests that pp105 represents the β-
subunit of an IGF-I receptor with an altered molecular weight.

To determine whether the increase of the molecular weight

![Fig. 6. Tryptic phosphopeptide mapping of 95-kDa phos-
phoprotein of human muscle (a) and 105-kDa phosphoprotein
of HL-60 cells (b). WGA-purified receptor from human muscle and
HL-60 cells was incubated with 100 nM/liter insulin, phosphorylated
with [γ-32P]ATP and prepared for HPLC tryptic phosphopeptide
mapping as described under “Experimental Procedures.”]
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When membrane glycoprotein extracts from HL-60 cells were immunoprecipitated with an insulin-receptor antibody which also cross-reacts with the IGF-I receptor protein we identified a protein pp105 which is larger than the β-subunits of insulin receptor (12, 13, 16–17, 22–25, 26, 36) and IGF-I receptor (8–10, 14, 37) in different tissues.

In addition, this protein is phosphorylated not only in tyrosine but also in threonine residues. Since the tryptic peptide map of insulin-stimulated pp105 is different from the peptide map of insulin-stimulated insulin receptor β-subunit, it is unlikely that pp105 is related to the insulin receptor. This conclusion is also supported by the marked insensitivity of pp105 phosphorylation in response to insulin, whereas a very high sensitivity in response to IGF-I is found. This high sensitivity to IGF-I strongly suggest that the protein which is recognized by our antibody represents an altered IGF-I receptor β-subunit. This is also in accordance with our cell culture studies in which we have identified low dosages of IGF-I as a potent stimulus of HL-60 cell growth.

Molecular weight heterogeneities of the α-subunit of insulin and IGF-I receptors of different tissues have been identified and found to be due to differences in oligosaccharide content (14, 36, 38). However, in these studies there was no evidence for a molecular weight difference in the β-subunit as observed in our experiments. We tried to deglycosylate the pp105 with neuraminidase and glycopeptidase F in order to determine whether the molecular weight increase of the pp105 is due to an increased oligosaccharide content. Only a slight (2 kDa) decrease in the molecular mass was observed, which indicates that the molecular mass increase is only partly due to different glycosylation.

A similar molecular weight increase of the β-subunit, in this case the insulin receptor (40), has been described for another malignant cell line (U-937 monocytes). However, there is no evidence that the β-subunits of erythrocytes and monocytes from non-leukemic donors possess an altered β-subunit (17, 41). These results and our studies raise the question whether the molecular weight increase is specifically associated with the malignant state of these cells, and whether there are consequences for cell proliferation and differentia-

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M Kellerer, B Obermaier-Kusser, B Ermel, U Wallner, H U Häring and P E Petrides

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