The type 1 insulin-like growth factor receptor (IGF-IR), activated by its ligands plays an important role in the growth of cells in at least three different ways: it is mitogenic, both in vivo and in vitro. The IGF-IR is also capable of inducing differentiation in a number of cell types, raising the question of how the same receptor can send two seemingly contradictory signals, one for growth and one for differentiation. Using 32D cells, which are murine hemopoietic cells, we show that the activated IGF-IR can induce differentiation along the granulocytic pathway in a manner similar to the granuloocyte colony-stimulating factor. We find that one of the major substrates of the IGF-IR, the insulin receptor substrate-1 inhibits IGF-I-mediated differentiation of 32D cells. In the absence of insulin receptor substrate-1, functional impairment of another major substrate of the IGF-IR, the Shc proteins, is associated with a decrease in the extent of differentiation. Although the end points of the respective pathways remain to be defined, these results show for the first time that IGF-I-mediated growth or differentiation of hemopoietic cells may depend on a balance between two of its substrates.

The type 1 insulin-like growth factor receptor (IGF-IR),1 activated by its ligands plays an important role in the growth of cells in at least three different ways: it is mitogenic, both in vivo and in vitro, it is quasiboligatory for transformation, and it can protect cells from a variety of apoptotic injuries (1). The IGF-IR can also induce differentiation in certain types of cells, notably myoblasts (2, 3), adipocytes (4), osteoblasts (5), and cells of the central nervous system (6–8). These apparently contradictory actions of the IGF-IR, and indeed of other growth factor receptors (growth promotion on one side and induction of differentiation on the other) are usually dismissed as due to the “cell context,” a somewhat unsatisfactory answer. It is self-evident that, at some point, the mitogenic and differentiation signals originating from the IGF-IR will diverge. The question we have asked in this investigation is whether these signals can already be separated at the level of the receptor itself or its immediate substrates. We have used as a model 32D cells (9), which are well characterized diploid murine hemopoietic cells. 32D cells have an absolute requirement for interleukin-3 (IL-3), and undergo apoptosis when IL-3 is withdrawn (10, 11). IGF-I or overexpression of the IGF-IR prevent or markedly decrease apoptosis caused by IL-3 withdrawal (12–16). Indeed, an overexpressed IGF-IR causes 32D cells to grow in the absence of IL-3 at least for several days (14, 16). Another interesting characteristic of 32D cells is that they are completely devoid of insulin receptor substrate-1 (IRS-1) and IRS-2 (12, 17, 18). While the IGF-IR, by itself, protects 32D cells from apoptosis, overexpression of the insulin receptor (IR) is not sufficient for the growth of these cells in the absence of IL-3 (17). However, the combined overexpression of the IR and IRS-1 renders 32D cells IL-3-independent (17).

We show here that 1) the IGF-IR can also induce differentiation of 32D cells, along the granulocytic pathway; 2) IRS-1, one of the major substrates of the IGF-IR, inhibits IGF-I-mediated differentiation; 3) mutations of tyrosine 950 and of tyrosines 1250/1251 in the C terminus of the IGF-IR, in the absence of IRS-1, cause a decrease in the extent of IGF-I-mediated differentiation; 4) when these three tyrosine residues are mutated, the phosphorylation of Shc, another major substrate of the IGF-IR, is impaired; 5) overexpression of Shc promotes differentiation of 32D cells, while a dominant negative mutant of the Shc protein partially inhibits differentiation; and 6) the inhibitory effect of IRS-1 on the differentiation of 32D cells is associated with changes in the Akt/p70 S6 kinase pathway. We suggest that, at least in the case of 32D cells overexpressing the IGF-IR, the outcome (i.e. growth or differentiation) depends on a balance between the signaling pathways originating from two of its substrates.

**EXPERIMENTAL PROCEDURES**

**Retroviral Transduction**—32D clone 3 cells were transduced with a murine leukemia virus-based retroviral vector system (19) to express the wild type IGF-IR or its various mutants or its substrates, IRS-1 and Shc. These constructs have been described in previous papers from one of our laboratories (16, 20–22). The retroviral vector stocks were generated with a transient expression system (23) and used to transduce 32D cells as described previously (24). The cDNAs were inserted either into retroviral transfer vector MSCV.neoEB (carrying the neomycin resistance gene) or MSCV.hpl, (carrying the hygromycin resistance gene), which were kindly provided by Dr. R. G. Hawley (University of Toronto, Canada) and are described elsewhere (25).

The Shc-Sh2 cDNA was constructed as described (26). Briefly, the 5′ portion of Shc cDNA, corresponding to amino acid residues 1–377, was deleted and substituted with a 15-base pair sequence containing an eukaryotic initiation site for translation (TAAAGCACTATGGGC). This construction was carried out by polymerase chain reaction (PCR), using a sense primer containing the above mentioned initiation translation site and a XhoI restriction site in its overhang and an antisense primer containing the TAG stop codon and an EcoRI restriction site in its
gested and gel-purified PCR fragment was then ligated into the vector and transformed into E. coli. After the digestion, the enzymes were heat-inactivated at 68°C for 15 min, and the vector was used to transform competent cells.

Mixed populations obtained by retroviral transduction were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 10% WEHI cell conditioned medium as a source of IL-3 and the required antibiotics for the selection of the transduced cells. To stabilize the expression of the fusion proteins, the culture medium contained 100 units/ml granulocyte colony-stimulating factor (G-CSF; Life Technologies), 100 units/ml antibiotics (Life Technologies), and 100 units/ml bovine serum albumin for 3 days.

RESULTS

Immunoprecipitation and Immunoblotting—Cells were incubated in serum-free medium supplemented with 0.1% bovine serum albumin for 3 h before stimulation with the indicated growth factors, 20 ng/ml IGF-I (Life Technologies), 100 units/ml granulocyte-colony-stimulating factor (G-CSF; Life Technologies) or 10% fetal bovine serum. Cell lysates were resolved directly or after immunoprecipitation by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Immunoprecipitation and immunoblotting procedures are described by Valentinis et al. (27). The phosphotyrosine blots were performed with an antiphosphotyrosine horseradish peroxidase-conjugated antibody (PY20; Transduction Laboratories). The IGF-I receptor was immunoblotted with an anti-a-subunit IGF-I receptor polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). IRS-1 was immunoprecipitated and immunoblotted with monoclonal anti-IRS-1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY). She proteins were immunoprecipitated or immunoblotted with a polyclonal (Santa Cruz Biotechnology) anti-Shc antibody, respectively. Grb2 was immunoblotted with a monoclonal anti-Grb2 antibody (Transduction Laboratories).

Phospho-Akt and Akt protein were detected using the PhosphoPlus Akt (Ser473) antibody kit (New England Biolabs), following the manufacturer's instructions. Phospho-p70 S6 kinase (Thr421/Ser424) and p70 S6 kinase protein were visualized with a PhosphoPlus p70 S6 kinase antibody kit, also from New England Biolabs, and also following the manufacturer's instructions.

Survival and Differentiation Analysis—32D cells and the derived mixed populations obtained by retroviral transduction were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 10% WEHI cell conditioned medium as a source of IL-3 and the required antibiotics for the selection of the transduced cells. The mixed populations were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). 10% WEHI cell conditioned medium as a source of IL-3 and the required antibiotics for the selection of the transduced cells.

The present observations stem from our previous finding that, at variance with the IR, an overexpressed IGF-IR protects 32D cells from apoptosis although these cells do not have IRS-1 (12, 17, 18). As mentioned above, the IR requires the combined overexpression of IRS-1 to promote growth of 32D cells in the absence of IL-3 (17). When 32D cells overexpress the IGF-IR, they not only survive but also grow in the complete absence of IL-3 (12, 14). We had noticed, however, that, after a period of rapid growth, the 32D IGF-IR cells stop growing and, after 4–5 days, begin to decrease in number, especially when the culture is not supplemented daily with IGF-I (see below). The period of reduced growth coincided with the appearance of differentiated 32D cells. We were intrigued by the finding that the IGF-IR can protect 32D cells from apoptosis, while at the same time inducing terminal differentiation, which usually results in cell death (28). We therefore decided to examine the effects of the IGF-IR on 32D cells in more detail, using transduction with appropriate retroviral vectors.

Fig. 1. Overexpression of the IGF-I receptor induces differentiation of 32D cells. 32D cells overexpressing the human wild type IGF-IR (32D IGF-IR), like the parental 32D cells, grow well in IL-3 and show no evidence of differentiation. After removal of IL-3, 32D IGF-IR cells were incubated in 10% serum supplemented with 50 ng/ml IGF-I or with G-CSF (100 units/ml). The number of surviving (surv) and differentiating (diff) cells was determined at various times thereafter. The right is the number of surviving cells and the percentage of differentiated 32D cells at the times indicated after IL-3 withdrawal (range indicated to the right of each bar). On the left is the morphology of these cells, either by Giemsa staining or after staining for myeloperoxidase. For comparison, we are also showing the levels of survival and G-CSF-induced differentiation in the parental 32D cells. Survival is expressed as the fraction of cells recovered in relation to the number of cells seeded. Differentiation is expressed as percentage of band and polymorphonuclear cells in the surviving cells. These experiments were repeated 3–5 times for each condition.

The Activated IGF-I Receptor Induces Differentiation of 32D Cells—The use of retroviral vectors (see "Experimental Procedures") gives a good efficiency of transduction of 32D cells (notoriously resistant to transfection by plasmids) and generates mixed populations, expressing, in the case of the IGF-IR, approximately 100,000 to 200,000 receptors/cell (see below). The use of mixed populations obviates the problem of clonal variation and, since the expression levels are high, also compensates for slight variations in individual cells. The levels of expression are also reasonably uniform in the mixed population, as one would expect from retroviral transduction (24). By flow cytometric analysis of cells stained with an antibody to the IGF-IR, the width of the peak is essentially the same as the width of the peak in a cloned cell line (data not shown). The mixed population expressing the wild type human IGF-IR is here designated as 32D IGF-IR. Fig. 1 shows that these cells survive after IL-3 withdrawal and, with the addition of IGF-I, can actually grow, as already reported for 32D cells transfected with an IGF-IR plasmid (12, 14). 32D IGF-IR cells differentiate all the way to granulocytes, reaching a level of 50% differentiated cells by day 6 after IL-3 withdrawal and IGF-I addition (Fig. 1). Omission of IGF-I results in cell death, despite the
IGF-I Receptor and Differentiation

Mutational Analysis of IGF-I-induced Differentiation of 32D Cells—We investigated several mutants of the IGF-IR to determine the specific residues that are necessary for the induction of differentiation. These mutants have been described in previous papers from one of our laboratories (20–22) and are listed in Fig. 2a, which gives the levels of expression of the IGF-I receptors in mixed populations of transduced 32D cells. For simplicity, we are showing the results obtained with an antibody to IRS-1 or IGF-IR was as indicated under "Experimental Procedures." See "Results" for explanation of cell lines. Molecular masses (in kilodaltons) of marker proteins are indicated.

The reason we chose to present the results is that the antibody to the α-subunit results is that the antibody to the β-subunit does not recognize the receptor truncated at residue 1245. Notice that the α-subunit of 1245 is of normal size, but the proreceptor is a little shorter, because of the truncated β-subunit. We have compared levels of expression with those of cell lines with a known number of IGF-IRs (30). We can say that all populations express at least 50,000 receptors/cell, which is more than it is needed for other functions of the IGF-IR (30).

Fig. 3a gives the survival at 4 days after IL-3 withdrawal of three of these mixed populations. The overexpressed mutant receptors, 32D 4 basic aa, 32D 6 serine, and 32D 1293–94 (Fig. 3a), are as effective as the wild type receptor in protecting 32D cells from apoptosis induced by IL-3 withdrawal, and all of them induce differentiation upon IGF-I addition (Fig. 3b). Other mutant receptors (32D 3YF, 32D d1245, 32D Y950, and 32D Y1250–51) were not capable of fully protecting 32D cells from apoptosis after the first 48 h. Since differentiation, even with the wild type receptor, does not become clearly visible until day 4, it was necessary to prolong the survival of these cell lines by the addition of 0.1% WEHI medium (hereafter designated as IL-3). This procedure partially decreases the extent of differentiation in 32D cells expressing the wild type receptor to about 18% of viable cells (Fig. 3d). The level of differentiation is roughly the same as that of parental 32D cells growing in IL-3 (see Fig. 1). It may be argued that these mutant receptors are nonfunctional receptors, incapable of transmitting an IGF-I-mediated signal. The addition of 0.1% IL-3 was not sufficient to induce survival in the cell line described in Fig. 3c (not shown). The addition of IGF-I made these cell lines grow with two exceptions: the parental cell line and the 32D 3YF cell line (Fig. 3c). The 3YF mutant receptor has been known to be an inactive receptor (31). The dependence on IGF-I for survival and growth of the 32D Y950, 32D Y1250–51, and 32D d1245 cell lines indicates that these mutant receptors are responsive to stimulation by IGF-I, as indeed they are when transfected into other cell lines (20–22). We can therefore say that these mutant receptors can still transmit a mitogenic signal but have lost the ability to transmit a differentiation signal.

It could be objected that 32D Y950, 32D Y1250–51, and 32D d1245 cell lines may have lost the ability to differentiate. We therefore treated these same cell lines with G-CSF (100 units/ml). The wild type receptor cells gave (day 4) 19.5% differentiated cells, 20.8% Y950, 16.3% Y1250–51, and 14.1% 32D. Therefore, these cell lines can differentiate when induced by G-CSF but not when induced by IGF-I.

Role of IRS-1 in IGF-I-induced Differentiation of 32D Cells—Since 32D cells are devoid of IRS-1 and IRS-2 (17), we inquired whether the absence of these proteins played a role in IGF-I-mediated differentiation of 32D cells. For this purpose, we examined other cell lines, all derived from the parental 32D cells: 1) a cell line in which we introduced, through a retroviral vector, the IGF-IR into a 32D cell line overexpressing IRS-1 (32D IRS/IGF-IR) (the original 32D IRS-1 cell line was a kind gift from Dr. Morris White and was described previously also from our laboratory (16)); 2) a cell line in which a retroviral vector expressing IRS-1 was introduced into 32D IGF-IR cells (in order to avoid confusion, these cells are designated as 32D GR15/IRS cells); and 3) a cell line overexpressing the IGF-IR and transduced with the empty retroviral vector used to deliver IRS-1 (32D IGF-IR/hph). Fig. 2b shows that IRS-1 is not detectable in the parental 32D cells (lane 1), in the 32D IRS-1 cells (lane 2), and in 32D IGF-IR/hph (lane 3). It is modestly expressed in 32D GR15/IRS cells (lane 4) and strongly overexpressed in the 32D IRS-1/IGF-IR (lane 5). This blot was purposely overexposed in order to demonstrate again the absence of IRS-1 from the parental 32D cells. The levels of the expression of the IGF-IR in these cell lines are also given in Fig. 2b. Notice the comparable level of the overexpressed receptor in all
four 32D-derived cell lines and the very low level of expression of the IGF-IR in parental 32D cells.

Table I shows a representative experiment on the survival and differentiation of these cell lines, in the absence of IL-3 but with the addition of IGF-1. The 32D IGF-IR cells and the same cells transduced with an empty retroviral vector for IRS-1 (32D IGF-IR/hph) survive after IL-3 withdrawal, grow, and differentiate (40–45% differentiated cells). The combined overexpression of IRS-1 and the IGF-IR (32D GR15/IRS and 32D IRS/IGF-IR) results in cell lines that also survive and grow extremely well in the absence of IL-3. These last two cell lines, however, do not differentiate as well as the cells expressing only the IGF-IR (Table I). The mixed population of 32D cells expressing the IGF-IR and moderate amounts of IRS-1 (32D GR15/IRS) had 8.9% differentiated cells; the one expressing high amounts of IRS-1 (32D IRS/IGF-IR) had 0.3%. The difference between these two cell lines expressing both the IGF-IR and different levels of IRS-1 will be discussed further below. Table I shows the extent of differentiation at day 4, but we examined these cell lines also at day 6, and the cell lines that did not differentiate remained undifferentiated even at day 6, when 50% of the 32D IGF-IR cells are already differentiated. It seems therefore, that IRS-1 inhibits IGF-I-induced differentiation of 32D cells. We have confirmed the observation that overexpressed IR, by itself, does not protect 32D cells from apoptosis but that the combined overexpression of the IR and IRS-1 makes these cells grow in the absence of IL-3 (17, 32). Interestingly, the 32D IR/IRS1 cells do not differentiate (data not shown).

Role of Shc Proteins in Differentiation of 32D Cells—The Shc proteins (33) are a second major substrate of the IGF-IR. The 46- and 52-kDa isoforms are strongly expressed in 32D cells, roughly 10 times the levels of expression in 3T3 cells (data not shown). The p66 isoform is not expressed in these cells (34). We transduced 32D cells with a retroviral vector carrying the Shc protein cDNA that codes for the 46- and 52-kDa forms (a kind gift of Dr. Joseph Schlessinger). Even with retroviral vectors, it was not easy to obtain 32D clones overexpressing Shc proteins, because the cells seemed to differentiate spontaneously even in the presence of IL-3. We finally obtained two clones that expressed levels of Shc roughly twice the endogenous levels (Fig. 4a) and that grew in 10% IL-3 at least for 2–3 months. The increase in expression is modest but was confirmed by densitometric measurements and may be due to the fact that Shc proteins are already strongly expressed in 32D cells. The 32D cells overexpressing Shc proteins differentiate rapidly even in the presence of 5% IL-3 (Table II), while under the same conditions the parental 32D cells have a percentage of differentiated cells of 1.3%.

Since it has been reported that dominant negative mutants of Shc proteins (26) can inhibit differentiation of other cell types (35, 36), we transduced in 32D IGF-IR cells a retroviral vector expressing the SH2 domain of the Shc proteins (see "Experimental Procedures"). This construct is poorly expressed, and previous authors (26) could only detect it after

TABLE I
Survival and differentiation of 32D-derived cell lines

| Growth factor | Survival | Differentiation |
|---------------|----------|----------------|
| 32D IGF-IR    | IGF-I    | 0.7            |
| 32D IGF-IR/hph| IGF-I    | 1.1            |
| 32D GR15/IRS  | IGF-I    | 22.2           |
| 32D IRS/IGF-IR| IGF-I    | 21.1           |

FIG. 3. Survival and differentiation of 32D cells expressing various mutants of the IGF-IR. Both survival and differentiation were determined at day 4 after IL-3 withdrawal. Survival is expressed as -fold increase (or decrease) over number of cells plated. A 1-fold increase means that the number of cells has doubled. Differentiation is expressed as in Fig. 1. a, survival of the indicated cell lines in 10% serum, no IL-3, plus IGF-1 (50 ng/ml). b, differentiation of the same cell lines under the same conditions. c, survival of the indicated cell lines in 10% serum plus 0.1% IL-3 and plus IGF-1 (50 ng/ml). d, differentiation under the same conditions.
immunoprecipitation. We were able to detect its expression on Western blots, but it was necessary to expose the blot longer than the time required for detection of the endogenous Shc proteins (Fig. 4b). Note that the endogenous Shc proteins are at the same levels in all three cell lines (Fig. 4b). Despite the modest expression, the Shc dominant negative mutant partially inhibits IGF-I-mediated differentiation (Table II). The experiments were repeated, and the cells expressing the SH2 domain of Shc consistently had a decreased extent of cell differentiation, which, however, was never abrogated.

Tyrosyl Phosphorylation of Shc Proteins—We have examined Shc phosphorylation in the cell lines expressing the mutant receptors that fail to induce differentiation of 32D cells (32D Y950 and 32D Y1250–S1). As expected, 32D IGF-IR cells, stimulated with IGF-1 (Fig. 5a), show a clear increase in Shc phosphorylation. As previously reported for the IR (37), the 52-kDa isoform is the one that is preferentially phosphorylated (lane 5); serum is less effective than IGF-I in inducing tyrosyl phosphorylation of Shc (lane 6). A co-precipitation of Grb2 with Shc in these two lysates is clearly visible, especially prominent in the 32D Shc clone 10 sample, which expresses the empty retroviral vector that was used for the transduction of the Shc dominant negative mutant.
larger amounts than the other. The results (Fig. 5b, right) show that Shc is tyrosyl-phosphorylated in both the 32D IGF-IR and the 32D GR15/IRS (comparatively low levels of IRS-1). In the cell line strongly overexpressing IRS-1, Shc phosphorylation is not increased by stimulation with IGF-1 (lanes 5 and 6). Moreover, Grb2 co-precipitation with Shc is detectable only in 32D IGF-IR/hph cells (no IRS-1) and in the cells expressing lower levels of IRS-1 (32D GR15/IRS). A marked reduction in Grb2 co-precipitation is visible in anti-Shc precipitates of 32D IRS/IGF-IR cells (high levels of IRS-1). Finally, and in agreement with the results of Yamauchi and Pessin (39), when IRS-1 is immunoprecipitated from the same lysates, Grb2 co-precipitates with the phosphorylated IRS-1 in larger amounts in 32D IRS/IGF-IR cells than in 32D GR15/IRS cells, as expected (Fig. 5b, left). The amounts of IRS-1 and Shc that were immunoprecipitated by the respective antibodies were monitored by blotting the membranes after stripping and were similar (not shown).

**IGF-I Receptor and Differentiation**

DISCUSSION

There are several novel findings in the experiments reported above that we think are of interest. 1) We show for the first time that an activated IGF-IR can induce differentiation of hemopoietic cells along the granulocytic lineage. 2) The absence of IRS-1 (which is missing in parental 32D cells) is crucial for differentiation. When IRS-1 is reintroduced into 32D IGF-IR cells, differentiation is inhibited, and the extent of inhibition is dependent on the levels of IRS-1 expression. 3) IGF-I-induced differentiation (in the absence of IRS-1) requires the C terminus of the receptor. 4) At least three residues are important for differentiation, the tyrosine residue at 950 and the two tyrosines at 1250/1251. 5) Shc phosphorylation is decreased in cells expressing the two mutant receptors that fail to induce differentiation (Y950F and Y1250F/Y1251F). 6) Overexpression of Shc proteins favors differentiation, while a dominant negative mutant of Shc partially inhibits IGF-I-mediated differentiation. 7) While the IGF-IR, by itself, can promote temporary growth of 32D cells, the cells eventually differentiate, whereas the 32D IGF-IR cells expressing IRS-1 can grow indefinitely in the absence of IL-3 and IGF-I addition. Interestingly, the IGF-IR, by itself, seems to be defective in the activation of the Akt/p70 S6 kinase pathway, in respect to cells also expressing IRS-1.

Although we acknowledge that the end points of these two processes remain to be defined, our results show for the first time that the stimulation of the same receptor can lead to different biological outcomes (growth or differentiation), depending on a balance between substrate levels (the “cell context”).

The finding that an activated IGF-IR can induce differentiation of hemopoietic cells along the granulocytic lineage is a novel finding, although Merchav et al. (41) did report that injections of IGF-I in animals increased granuloopoiesis. The induction of differentiation is not trivial, according to the literature about on the same level as differentiation induced by G-CSF (29), and, as in the case of G-CSF (11), eventually results in a decrease in cell number. Thus, although we started out to resolve an ambiguity of the IGF-IR (cell proliferation versus differentiation), we find ourselves with another ambiguity. The IGF-IR promotes cell survival in IL-3-dependent cell lines (12–16, 22) and yet, at the same time, induces a differentiation program that eventually leads to cell death. Incidentally, IGF-I-mediated survival in the absence of IRS-1 and decreased Akt phosphorylation is due to the ability of the IGF-IR to use two alternative pathways for BAD phosphorylation; one pathway goes through the activation of mitogen-activated protein kinase, and a third one depends on the integrity of serine 1283, which results in the mitochondrial translocation of Raf-1.3

The number of receptors is reasonably uniform in our cell

---

2 M. Dews and R. Baserga, manuscript in preparation.

3 F. Peruzzi, M. Prisco, M. Dews, P. Salomoni, E. Grassilli, B. Calabretta, G. Romano, and R. Baserga, submitted for publication.
IGF-I Receptor and Differentiation

expresses large amounts of IRS-1 (see Fig. 2). In other words, it is possible that differentiation may occur by default, when 32D cells are stimulated to proliferate, but the mitogenic stimulus is not sustained. In favor of this interpretation are the findings of Fig. 6, discussed above. However, we believe that the situation is more complicated, and that a positive signal may also be required for the induction of differentiation.

There are several reasons for this belief. In the first place, IGF-I-induced differentiation of 32D cells is dependent on the presence of specific residues of the IGF-IR; these include Tyr950 and the tyrosine residues at 1250/1251 (see also below). These mutant receptors are functional, since they can transmit an IGF-I-mediated mitogenic signal (Fig. 3c), as we have already demonstrated in mouse fibroblasts (21, 57, 58). In fibroblasts, we also have shown that these mutant receptors protect from anoikis (58), an assay that, unfortunately, cannot be carried out in 32D cells. Their inability to induce differentiation is independent of their mitogenic capacity.

Second, Shc proteins seem to favor differentiation. True, it has been difficult to obtain strong expression of Shc proteins in 32D cells, which already express high levels of the 46- and 52-kDa isoforms. But even a modest overexpression seems to increase the tendency of these cells to differentiate. In addition, a dominant negative mutant of Shc partially inhibited IGF-I-mediated differentiation. A role of Shc in differentiation has also been suggested in PC12 cells induced by NGF (35) and in 32D cells induced to differentiate by thrombopoietin (36). We did not detect the 66-kDa isoform of Shc in 32D cells, confirming previous results in the literature (34).

There are other findings that relate the Shc proteins to differentiation by IGF-I. As mentioned above, Tyr950 and Tyr1250/Tyr1251 seem to be necessary for the differentiation process. The 1250/1251 residues of the IGF-IR have been reported to bind a protein (59), although the relevance of this protein to the differentiation process remains to be established. More important is perhaps the fact that a mutation at Tyr1250 markedly decreases, but does not abrogate, the internalization of the IGF-IR (57), and recent reports (37, 60) have shown that the IGF-1 and insulin receptors have to be internalized to phosphorylate Shc. This could explain why Shc phosphorylation by the 1250/1251 mutant IGF-IR is only decreased instead of being completely abolished, as in the case of the Tyr950 mutant. If this interpretation is correct, the requirement for 1250/1251 residues would be secondary to its effect on internalization, and the really crucial residue for differentiation would then be Tyr950 and its ability to phosphorylate Shc. Indeed, the 32D cells expressing the Y950F mutant receptor completely fail to phosphorylate Shc. Admittedly, the evidence for Shc inducing or favoring differentiation is circumstantial; the results are not clear cut, since we have partial inhibition by a dominant negative and partial inhibition of Shc phosphorylation by the 1250/1251 mutant. Perhaps another event is necessary that we have not yet identified. Most important, however, is the notion that any differentiating effect of Shc depends on the absence of IRS-1, whose overexpression simply overwhelms any tendency to differentiation.

The Shc proteins have also been thought to regulate the mitogenic process initiated by either insulin or IGF-1 (61, 62). We can speculate upon the relative proportions of IRS-1 and Shc proteins in determining mitogenesis or differentiation, a hypothesis that was indirectly suggested by the work of Yamachuchi and Pessin (39). In agreement with them (39), as already mentioned, the amount of Grb2 that co-precipitates with either IRS-1 or Shc depends on the levels and phosphorylation status of these substrates.

It will be an interesting pursuit to determine where the IRS-1 and Shc pathways diverge and converge. A clue may be
given by reports that the interactions of the IGF-IR with IRS-1 and Shc, although both centered at Tyr^305, differ in the requirements for the surrounding amino acids (50, 63) and that IRS-1 may also bind directly to the tyrosine kinase domain (50). Another intriguing aspect of this investigation is the relationship between survival and terminal differentiation, which is known to result in cell death.

In conclusion, using 32D cells, we have established that the IGF-IR induces granulocytic differentiation of murine hematopoietic cells and that the outcome (differentiation versus proliferation) may depend on a balance between two of its signaling pathways, IRS-1 inhibiting differentiation, and Shc favoring it. It seems reasonable to conclude that, in the case at least of the IGF-IR, “cell context” in determining growth or differentiation may depend on specific domains of the receptor and the availability of different substrates.

Acknowledgments—We are grateful to Dr. R. G. Hawley for providing the murine stem cell virus vectors and to Dr. A. J. Kingsman for providing the pHT vectors; to J. Verdone for skilled technical support; and to B. Vega for secretarial assistance.

REFERENCES

1. Baserga, R., Hongo, A., Rubini, M., Prisco, M., and Valentini, B. (1997) Biochem. Biophys. Acta 1332, 105–126
2. Flornini, J. R., Ewton, D. Z., Falen, S. L., and van Wyk, J. J. (1986) Am. J. Physiol. 250, C717–C778
3. Schmid, C., Steiner, T., and Froesch, E. R. (1984) FEBS Lett. 161, 117–121
4. Smith, P. J., Wise, L. S., Berkowitz, W., Wan, C., and Rubin, C. S. (1988) J. Biol. Chem. 263, 9402–9408
5. Schmid, C., Steiner, T., and Froesch, E. R. (1984) FEBS Lett. 173, 48–52
6. McCormis, F. A., Smith, T. M., Desalvo, S., and Furlanetto, R. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 822–826
7. Mill, J. F., Chao, M. V., and Ishii, D. N. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7120–7126
8. Recio-Pinto, E., Laff, F. F., and Ishii, D. N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2562–2566
9. Greenberger, J. S., Saakanyan, M. A., Humphries, R. K., Eaves, C. J., and Eckner, R. J. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2931–2935
10. Askew, D. S., Ashmun, R. A., Simmons, B. C., and Cleveland, J. L. (1991) Oncogene 6, 1915–1922
11. Metcalf, D. (1985) Blood 65, 357–362
12. Dews, M., Nishimoto, I., and Baserga, R. (1998) Recept. Signal Transduc. 7, 231–239
13. McCubrey, J. A., Stillman, L. S., Mayhew, M. W., Algate, P. A., Dellow, R. A., Avruch, J. (1995) J. Biol. Chem. 270, 23464–23468
14. Bogenberger, J., and Blom, J. (1997) J. Cell. Sci. 110, 1515–1519
15. You, R., and Cooper, G. M. (1996) Mol. Endocrinol. 10, 839–842