Stimulation by Insulin-like Growth Factors Is Required for Cellular Transformation by Type β Transforming Growth Factor*

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Medium conditioned by BRL-3A cells, a known source of insulin-like growth factor II (IGF-II), induced phenotypic transformation (anchorage-independent proliferation) of mouse BALB/c 3T3 fibroblasts but not rat NRK-49F fibroblasts, in the presence of 10% calf serum. A specific radioreceptor assay and a bioassay indicated that BRL-3A conditioned medium contained 0.5—1 ng/ml of type β transforming growth factor (βTGF). Purified IGF-II and βTGF acting together reconstituted the transforming activity of BRL-3A conditioned medium on BALB/c 3T3 cells. Insulin was 5—10% as potent as IGF-II in supporting the transformation action of βTGF on BALB/c 3T3 cells. NRK-49F cells were phenotypically transformed by βTGF in the presence of EGF and 10% calf serum as the sole source of IGFs. However, transformation of NRK-49F cells under these conditions was inhibited by addition of purified IGF-binding protein. Addition of an excess of IGF-II prevented the inhibitory action of IGF-binding protein. The different sensitivities of the two cell lines to IGFs was correlated with lower levels of type I IGF receptor and higher levels of type II IGF receptor in NRK-49F cells as compared with BALB/c 3T3 cells. The results suggest that cellular stimulation by IGFs is a prerequisite for transformation of rodent fibroblasts by βTGF. We propose that transformation of fibroblasts by βTGF requires concomitant stimulation by the set of growth factors that support normal cell proliferation.

Transforming growth factors (TGFs) are hormonally active polypeptides that induce the transformed phenotype when added to fibroblasts in culture (1). TGFs were initially found after observing that serum-free medium conditioned by oncogenically transformed cells was capable of inducing anchorage-independent proliferation of normal NRK rat fibroblasts (1). Two distinct types of TGFs from these culture fluids are responsible for the induction of the transformed phenotype in NRK cells (2, 3). The first type, αTGF, is a 6-kDa polypeptide that exhibits significant amino acid sequence homology with epidermal growth factor (EGF) (3—6). αTGF binds to cellular receptors for EGF and activates a receptor-associated tyrosine protein kinase with a potency similar to that of EGF (1, 7, 8). The second type of TGF, βTGF, is a disulfide-linked dimer of two very similar or identical chains of 12 kDa each (9—12). βTGF has been purified to homogeneity from transformed (12) as well as normal sources (9—11) and is abundant in blood platelets (10, 13). βTGF interacts with high-affinity cell surface receptor complexes that contain a ligand-binding subunit of 280—330 kDa (14).

The observation that αTGF and βTGF acting separately do not induce phenotypic transformation of NRK fibroblasts led to the hypothesis that a unique form of synergism exists between the cellular effects of these two factors (2, 3, 9—12). The ability of EGF to substitute for αTGF in potentiating the transforming action of βTGF added support to this hypothesis. However, more recent work has suggested that growth factors present in serum may also play a role in the action of TGFs (15, 16). Specifically, platelet-derived growth factor (PDGF) present in calf serum routinely added to anchorage-independent proliferation assays was found to be required for the transforming action of EGF and βTGF on NRK cells (17). In this communication, we report that insulin-like growth factors (IGFs) from calf serum or other sources are also required for the expression of the transformed phenotype in rodent fibroblasts. Since the set of growth factors, PDGF, EGF, and IGFs, required for the induction of the transformed phenotype by βTGF are those which act in concert to stimulate normal proliferation of fibroblasts in culture (18, 19), we propose that full mitogenic stimulation is a prerequisite for the expression of the transforming action of βTGF.

EXPERIMENTAL PROCEDURES

Serum-free Waymouth's medium was conditioned by confluent monolayers of BRL-3A cells as described before (20). Conditioned medium was collected every other day and 10 consecutive collections obtained from the same culture were pooled and used in these experiments. IGF-II from serum-free medium conditioned by BRL-3A cells, βTGF from human platelets, and EGF from mouse submaxillary glands were purified to homogeneity by published procedures (10, 14, 20, 21). Human IGF-I (from Dr. M. Czech, University of Massachusetts) was originally obtained from Dr. R. Humbel (University of Zurich). Porcine insulin was a gift of Dr. R. Chance (Eli Lilly).

Stock cultures of BALB/c 3T3 mouse fibroblasts (American Type Culture Collection) and NRK-49F rat fibroblasts (from Dr. J. De Larco, National Cancer Institute) were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, or with 5% calf serum plus nonessential amino acids, respectively.

Soft agar assays were performed as described before (1, 3) in the presence of 10% calf serum (Gibco, lot 29K6321) plus the growth factors indicated in each experiment. Assays were performed in duplicate, and read unfixed and unstained 10 days later. At least 200 cells or colonies were counted per assay. Colonies over 40 μm in diameter (containing over 50 cells/colony) were scored as positive. Results are expressed as the per cent of cells developing colonies after 10 days in culture.

βTGF, IGF-I, IGF-II, and insulin were labeled with 125I as described before (14, 22). The βTGF radioreceptor assay consisted of measuring

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The abbreviations used are: TGF, transforming growth factor; αTGF, βTGF, type α and type β TGFs, respectively; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor.

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the ability of samples to compete with 1.25 ng/ml of 125I-βTGF for binding to specific receptors (14) in confluent monolayers of BALB/c 3T3 cells in 35-mm culture wells. All samples to be assayed were lypophilized and were added to the assay resuspended in 1.5 ml of binding buffer (14). Total binding of 125I-βTGF to the monolayers in the absence of competitors was 1700 cpm/well. Nonspecific binding, determined in the presence of 250 ng/ml of βTGF was 710 cpm/well. Half-maximal displacement of 125I-βTGF from the receptor was effected by 2.75 ng/ml of βTGF. One βTGF ng eq is defined as the amount of competing activity which causes the same amount of competition in the radioreceptor assay as 1 ng of purified platelet βTGF.

IGF-binding protein was purified to homogeneity from serum-free Dulbecco’s modified Eagle’s medium conditioned by H-35 rat hepatoma cells. Details of the purification will be published elsewhere. Briefly, the conditioned medium was dialyzed against 0.1 M acetic acid, lyophilized, and chromatographed over a Bio-Gel P-10 column in the presence of 1 M acetic acid. Fractions containing 125I-IGF-II binding activity were pooled, and IGF-binding protein was extracted from this sample by binding to, and elution from, IGF-II immobilized on CNBr-activated Sepharose 4B (Pharmacia). The resulting product contained one major protein species, 36.5 kDa, as determined by dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of the gels.

Affinity labeling of IGF and insulin receptors in BALB/c 3T3 and NRK-49F cells was done with the respective radioactive ligands and disuccinimidyl suberate, as described before (22-24).

RESULTS AND DISCUSSION

Serum-free media conditioned by various cell lines in culture were screened for their ability to induce anchorage-independent proliferation (colony growth) of rodent fibroblasts in semisolid medium. We observed that medium conditioned by BRL-3A rat liver cells, a known source of IGF-II, was able to induce colony growth of 3T3 mouse fibroblasts but not NRK rat fibroblasts, in the presence of 10% calf serum (Fig. 1). NRK cells are an indicator cell type extensively used for the characterization of TGFs. They are very sensitive to the transforming action of EGF or αTGF plus βTGF, in the presence of calf serum (1-3, 8-12). Addition of exogenous βTGF to soft-agar assays containing BRL-3A conditioned medium plus calf serum did not induce colony growth of NRK cells (not shown), but addition of exogenous EGF did (Fig. 1). These observations suggested the presence of βTGF, but not EGF or αTGF, in medium conditioned by BRL-3A cells.

That serum-free medium conditioned by BRL-3A cells contains βTGF in addition to IGF-II was documented by a specific radioreceptor assay after fractionation of this medium over Bio-Gel P-10 molecular filtration columns (Fig. 2). A peak of βTGF receptor-competing activity eluted in a position corresponding to 10-14 kDa proteins. Furthermore, the fractions containing radioreceptor competent material also exhibited βTGF bioactivity as defined by the ability to induce strong anchorage-independent proliferation of NRK cells in the presence of exogenous EGF (Fig. 2). The eluting position of the bioactivity was that peculiar to βTGF from normal and transformed sources, a 25 kDa polypeptide that is anomalously retarded by the Bio-Gel chromatography matrix (9, 10, 12). This peak of colony-forming activity was preceded by another peak of biologically active material. The nature of the second peak is unclear. It is possible, however, that all the bioactivity seen in Fig. 2 corresponds to βTGF coeluting with an inhibitor of its action in fractions 47-53 of the chromatography. A similar elution profile with a split peak of βTGF bioactivity is caused by the presence of a coeluting inhibitor, identified as spermidine, during purification of βTGF from human placenta and bovine lung and kidney (9, 25). Based on the results of the radioreceptor assay, we estimate that BRL-3A conditioned medium contains 0.5-1.0 ng/ml of βTGF.
Homogeneous preparations of various growth factors were used to investigate what components of BRL-3A cell-conditioned medium were responsible for the induction of the transformed phenotype in 3T3 cells. EGF and βTGF assayed separately displayed little or no activity. Together, they induced a proliferative response in 3T3 cells not as extensive as in NRK-49F cells (Fig. 1). These data suggested that the ability of 3T3 cells to respond to BRL-3A conditioned medium was not likely due to high sensitivity to minute amounts of EGF analogues possibly present in that medium. βTGF purified to homogeneity from BRL-3A medium was unable to induce colony growth when assayed alone. However, when IGF-II and βTGF were assayed together, they reconstituted the transforming activity of BRL-3A cell-conditioned medium on 3T3 cells (Fig. 1). Porcine insulin was able to substitute for IGF-II in this action (Fig. 1), but IGF-II was 10–20 times more potent than insulin on a molar basis (Fig. 3). These data indicate that IGF-II is involved in the induction of the transformed phenotype by βTGF in 3T3 cells and that this effect of IGF-II is likely mediated by IGF receptors with which insulin cross-reacts weakly (22, 26).

In contrast to 3T3 cells, NRK cells did not require supplementation with purified IGF-II to proliferate in semisolid medium in the presence of βTGF, EGF, and calf serum. However, the following experiments, summarized in Table I, strongly suggested that calf serum present in the assays provided IGFs required for the response of NRK cells to βTGF. To test the role of IGFs from calf serum in the induction of the transformed phenotype by βTGF, NRK cells were plated in semisolid medium in the presence of βTGF, EGF, and 10% calf serum with or without 2 μg/ml of purified IGF-binding protein.6 IGF binding protein strongly inhibited the anchorage-independent proliferative response of NRK cells (Table I). Addition of an excess IGF-II to the assays prevented the inhibitory action of IGF binding protein (Table I). These results suggest that IGF-binding protein inhibited colony growth of NRK cells by decreasing the concentration of free IGFs supplied by calf serum in the assays.

We conclude that, like 3T3 cells, NRK cells require stimulation by IGFs to respond to βTGF in soft-agar assays. The concentration of IGF needed to provide this stimulation in NRK cells is, however, lower than in 3T3 cells. A possible basis for this different sensitivity to IGFs may be the different levels of type I and type II IGF receptors found in NRK and 3T3 cells. Thus, when relative receptor levels in these two cell lines were estimated by receptor affinity labeling with 125I-IGF-I and 125I-IGF-II it was found that NRK cells had markedly higher levels of type II IGF receptors and lower levels of type I IGF receptors than 3T3 cells (Fig. 4). No significant difference was observed in the levels of insulin receptors in these two cell lines (Fig. 4).

In addition to the well-known requirement for EGF, or αTGF (2–12), and the requirement for IGFs as shown here, induction of anchorage-independent proliferation by βTGF also requires PDGF. Thus, Assouan et al. (17) showed that EGF and βTGF induce colony growth of NRK cells in the presence of calf serum but not platelet-poor plasma unless exogenous PDGF is added. PDGF, EGF, and IGFs acting in sequence are known to provide the necessary stimuli for full mitogenic stimulation of rodent fibroblasts in culture (18, 19). These three types of growth factors are present in calf serum (18, 19), which generally at 10% concentration supports the

![Fig. 3. Concentration dependence of the effect of IGF-II and insulin on the induction of the transformed phenotype in 3T3 cells by βTGF. The indicated concentrations of IGF-II and insulin were added to soft-agar assays containing 0.1 nM platelet βTGF plus 10% calf serum, and 3T3 cells as the indicator cell type. Data are the average of duplicate determinations.](image)

**TABLE I**

| Conditions* | Anchorage-independent proliferation |
|-------------|-----------------------------------|
|             | % colonies*                        |
| Control     | 74                                |
| + IGF-II    | 70                                |
| + IGF-binding protein | 24                              |
| + IGF-binding protein + IGF-II | 59                              |

*Anchorage-independent proliferation of NRK-49F cells was assayed in the presence of 0.1 nM βTGF plus 0.3 nM EGF and 10% calf serum alone (control) or with 0.1 μM IGF-II and/or 2 μg/ml of IGF-binding protein, as indicated.

*Assays done in duplicate gave values that varied by less than 9%. Data are the average of duplicate values. The experiment was repeated twice with similar results.

![Fig. 4. Relative levels of IGF and insulin receptors in NRK and 3T3 cells affinity labeled with 125I-IGF-I, 125I-IGF-II, and 125I-insulin. NRK and 3T3 cells were detached from culture vessels by brief exposure to buffer containing 1 mM EDTA. After washing, aliquots (2 × 10⁶ cells in 0.5 ml) of the resulting cell suspensions in binding buffer were incubated for 90 min at 10 °C in the presence of 2 nM radiolabeled ligands alone or with 0.5 μM IGF-II or 1 μM insulin, as indicated. Cells were then washed in the cold and cross-linked with cell-bound ligands by exposure for 15 min at 0 °C to 0.15 mM disuccinimidyl suberate. Cross-linking reactions were arrested by solubilization of cells in the presence of 1% Triton X-100 in 10 mM Tris buffer, and electrophoresis on 6% polyacrylamide gels in the presence of sodium dodecyl sulfate and dithiothreitol (27). Autoradiograms (6 days) from the resulting fixed, dried gels are shown. The specifically labeled receptor bands are indicated. Type I IGF receptors migrate as a labeled, free α subunit (α Type I-R) of 135 kDa in the presence of reducant (22), and a 270-kDa labeled protein product proposed to consist of two α subunits cross-linked together. Characteristically, labeling of these receptor species is inhibited by an excess of unlabeled IGF-II which cross-reacts with type I IGF receptors (22). Type II IGF receptors (Type II-R) are characterized by a 250-kDa receptor species specifically labeled by 125I-IGF-II, and to a lesser extent by 125I-IGF-I (22). Affinity-labeled insulin receptors characteristically migrate as a specifically labeled, free α subunit (α Ins-R) of 125 kDa in the presence of reducant (24). K, kilodalton.](image)
proliferation of fibroblasts in monolayer culture. Ten per cent calf serum is routinely present in the soft-agar assays for TGFs performed by us and others (1–3, 9–12). However, this concentration of calf serum alone or supplemented with additional EGF or IGFs does not support proliferation of rodent fibroblasts in soft agar unless OTGF is present. Therefore, we conclude that full mitogenic stimulation of rodent fibroblasts is a prerequisite for the induction of the transformed phenotype by PTGF. The need for additional EGF, aTGF, or IGF in soft-agar assays would only reflect a decreased sensitivity or accessibility of the cells to individual growth factors in semisolid medium, rather than unique forms of synergism between aTGFs or IGFs and PTGF as previously proposed.

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