An Agent or Agents Produced by Virus-transformed Cells Cause Unregulated Ruffling in Untransformed Cells

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Abstract. KNRK cells (a normal rat kidney [NRK] cell line transformed by Kirsten murine sarcoma virus) in sparse culture exhibit a highly ruffled morphology, but the cause of this ruffling is unknown. In this study, we have demonstrated that the continuous, excess ruffling on KNRK cells is caused by one or more soluble agents secreted by the KNRK cells themselves. To do this study, an assay for ruffling responses in live cell cultures was defined, and its reproducibility was demonstrated. This assay permitted observation of the kinetics of ruffling responses (percentage of cells ruffled as a function of time after stimulation). This method was used to compare the kinetics of ruffling induced by insulin, epidermal growth factor, fibroblast growth factor, glucose, and KNRK cell conditioned medium (CM). Ruffling was elicited on NRK cells by each of the polypeptide mitogens and nutrients, but, in each case, this ruffling subsided spontaneously within an hour. CM from KNRK cells also caused ruffling movements on untransformed NRK cells, but this ruffling continued for at least 20 h. This response was largely blocked by premixing the KNRK cell CM with rabbit IgG against rat transforming growth factor, type α, (TGF-α). KNRK cells made quiescent (ruffle free) by a pH shift (from 7.4 to 8.4) responded to insulin, glucose, and KNRK cell CM with kinetics similar to those observed for each of these factors in NRK cells. The unusual feature for the ruffle-inducing agent(s) produced by KNRK cells was that this activity was not subject, in either NRK or KNRK cells, to the cellular off-regulation that limits the responses to insulin or glucose. Thus, the continuous ruffling of KNRK cells is caused by their own unregulated ruffle-inducing agent or agents, which appear to include TGF-α. This work also demonstrates that kinetic analysis of cellular responses to exogenous factors can provide new insights into the regulatory mechanisms involved in the normal limitation of these responses.

RUFFLING on the free cell margins of sparsely cultured cells can be elicited by various hormone-like growth factors including epidermal growth factor (EGF)[1] (7, 8, 14, 16), nerve growth factor (8), platelet-derived growth factor (28), and even fresh serum-containing medium. With each factor, ruffling appears to subside spontaneously in <1 h at 37°C, indicating cellular off-regulating mechanisms. Kinetic analysis of these responses is limited by most methods used to observe ruffling. Scanning electron microscopy provides excellent detail in the ruffles themselves but does not permit repeated observations on the same set of cells over an experimental time course and is too cumbersome to confirm repeatedly kinetic observations under numerous experimental conditions. An alternative technique, time-lapse cinematography, permits elegant visualization of ruffling activities over time on the same cells, but the number of cells that can be observed at the required high magnification is too limited to permit extensive experimentation. The most useful approach to the analysis of ruffling kinetics is to observe living cells directly with the aid of inverted phase microscopy. Goshima et al. (13) used this direct method to observe the ruffling induced by insulin treatment of KB cells. By repeatedly counting the percentage of ruffled cells in the same specimen over a period of time after the addition of insulin, these authors demonstrated the transient kinetics of insulin-induced ruffling at several concentrations and at several temperatures. They also observed that glucose caused a small ruffling response, but the duration of that response was not reported.

Increased and continuous ruffling is a frequently observed correlate of malignant transformation (1, 19, 21). Neither the molecular mechanism underlying this alteration nor the significance of it is known. In particular, it is not known how or if the ruffling of transformed cells relates to the transient mitogen-induced ruffling of normal cells. In the present study, we compared ruffling responses in a normal rat kidney cell line (NRK) and a line of Kirsten murine sarcoma virus–transformed cells (KNRK cells). Our objective was to determine the cause of the apparently unregulated (not transient) ruffling observed in the transformed line. Several polypeptide mitogens (insulin, EGF, and fibroblast growth factor) and low molecular weight nutrients (glucose and vitamins) were tested for ruffle-inducing capacity. The following factors were studied extensively: Insulin was chosen as a representative poly-

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; KNRK, Kirsten murine sarcoma virus–transformed NRK; NKR, normal rat kidney; TGF, transforming growth factor; TRA, tumor ruffling agent.

peptide mitogen because several other early insulin effects, including increased hexose (9, 12, 17) and amino acid transport (13), have been described. Glucose was chosen because it is a simple, chargeless nutrient for which membrane transport systems have been extensively studied, transport is augmented after insulin stimulation or as a component of the transformed phenotype (10, 15, 32), and ruffle-inducing capacity has been reported (13). In addition, we tested conditioned buffer from both transformed and untransformed cells. We considered and tested four independent hypotheses which together appear to exhaust the potential alternative explanations for this phenomenon: (*a*) Glucose (or nutrient)-induced ruffling is not subject to off-regulation as is the ruffling induced by peptide mitogens, and KNRK cells have an enhanced glucose ruffling response. (*b*) KNRK cells no longer require any stimulation to ruffle. (*c*) KNRK cells have lost the cellular off-regulatory control of responses to exogenous factors. (*d*) KNRK cells produce their own ruffle-inducing factor or factors that are not off-regulated.

It was important to be able to observe, at brief intervals, the individual effects of a series of treatments and to monitor both the onset and the cessation of ruffling within a specific specimen. To do that, we used the direct observation method of Goshima et al. (13), first testing to ensure that our observation methods gave valid and reproducible results.

Materials and Methods

NRK (11) and KNRK (25) cells were cultured in sparse monolayers (10% fetal bovine serum, Waymouth's medium MB752/1, 37°C, 5% CO_2 atmosphere). On day 2 after passage, complete medium was replaced with nutrient deficient Waymouth's medium, made without glucose, amino acids, and vitamins (i.e., a buffer with the same salt composition as Waymouth's medium BM752/1). Complete Waymouth's medium with or without fetal bovine serum was used in indicated experiments. After 2–4 h, cells were examined by inverted phase microscopy using a 25X objective lens for total magnification of ~250. Ruffling was quantified by rapidly scanning visually several areas within each culture dish, counting numbers of cells with and without marginal ruffles, and recording the percentage of cells ruffled. Approximately 100 cells were counted for each data point. A cell was considered ruffled if more than ~2–3% (by visual estimate) of the free margin was ruffling. Ruffling was scored just before and at brief intervals after addition of the ruffling agent. For each type of agent, specificity of ruffling was tested by the addition of an equal volume of blank carrier with the same mixing techniques. This ensured that mechanical factors or slight changes in pH or salt concentration had not caused the observed ruffling. Insulin was from Sigma Chemical Co., St. Louis, MO (bovine pancreas crystalline, I-5500), epidermal growth factor and fibroblast growth factor were from Collaborative Research Inc., Lexington, MA); vitamins were a mixture of the vitamins in Waymouth's medium MB752/1 as described by Gibco (Grand Island, NY). Antiserum to TGF-α was a gift from Dr. Daniel R. Twardzik (Oncogen, Seattle, WA) (18).

Results

Transient Response to Insulin, EGF, Fibroblast Growth Factor, Glucose, or Vitamins

Sparse cultures of untransformed NRK cells in complete medium exhibited minor (~5% of free cell margin) ruffling on 10–40% of cells. After 2 h in nutrient-deficient Waymouth's medium (hereafter called buffer), only 5–10% of NRK cells were ruffled. The addition of 25 μg/ml insulin rapidly produced ruffles on most cell margins (Fig. 1, *a* and *b*). Individual ruffles appeared and disappeared (Fig. 1, *b–d*), but after an hour, most ruffles were gone (Fig. 1 *e*), even in the continuous presence of insulin. To quantify the kinetics of this response, the percentage of cells ruffled was counted repeatedly on the same culture dish. The numbers of cells counted had to be large enough to give reproducible results, yet the counts had to be rapid enough to observe fast changing values. For this purpose, several fields of 15–20 cells each were observed for a total of ~100 cell/count, and percent ruffled cells plus field-field variation was noted. This method produced reproducible kinetic curves. However, because values changed rapidly and observations were not done at exactly the same time points from experiment to experiment, mean values could not be calculated. Therefore, to compare repetitions of the same experimental condition, individual curves were overlaid on each other atop a light box. From this,

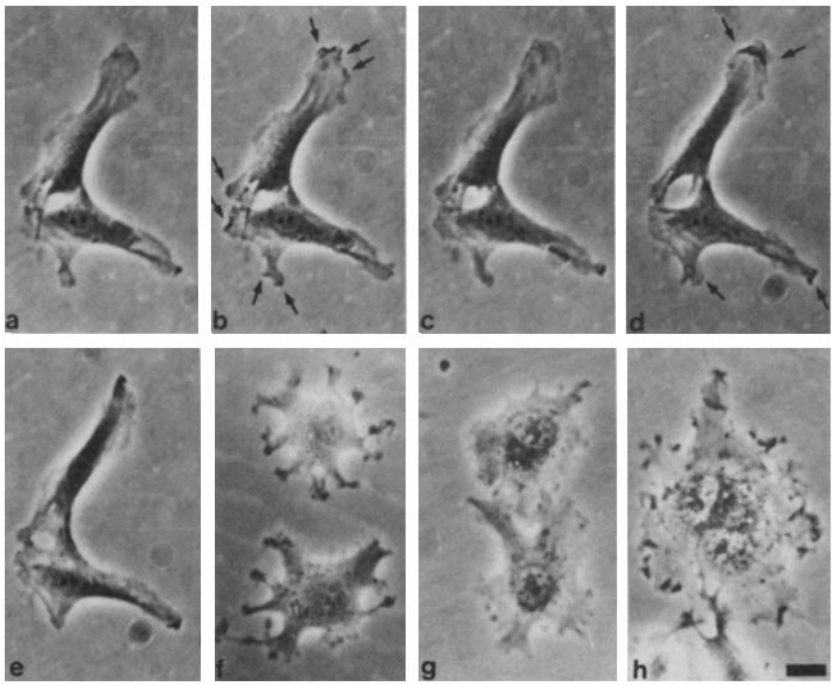

Figure 1. Phase-contrast appearance of quiescent and ruffled cells, which are quantified in Figs. 2–5. (*a*) Two quiescent NRK cells after 2 h equilibration in pH 7.4 buffer (nutrient deficient Waymouth's medium; see Materials and Methods); (*b*) same cells as in *a* 3 min after insulin treatment. Arrows indicate ruffles, observed as dark lines. More ruffles per cell are seen by fine focusing and with observation of actual movement; (*c*) same cells 10 min after insulin. Ruffles on these two cells were temporarily diminished, whereas many other cells in the preparation were ruffled; (*d*) same cells 38 min after insulin. These cells were again ruffling (arrows), others in the preparation were quiescent; (*e*) same cells 60 min after insulin, little ruffling on these or other cells in this preparation. (*f*) Highly ruffled KNRK cells after 3 h in pH 7.4 buffer. (*g*) Quiescent KNRK cells after 3 h in pH 8.4 buffer. (*h*) KNRK cells first made quiescent by equilibration in pH 8.4 buffer then treated with insulin for 4 min to produce ruffles (Bar, 10 μm).

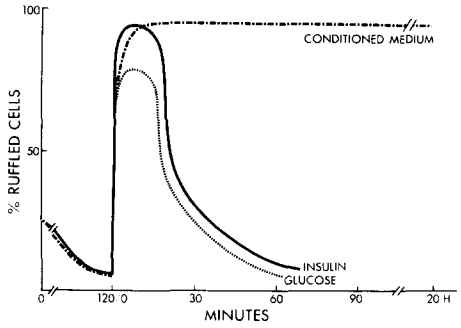

Figure 2. Percent of NRK cells ruffled after exposure to insulin, glucose, or KNRK conditioned medium as a function of time. Curves marked insulin and glucose: Cells were equilibrated for 2 h in buffer, then exposed to insulin or glucose. Insulin (final concentration 25 μl/ml) was added as a bolus in 0.005 N HCl and swirled gently. (The same volume of carrier alone did not produce significant ruffling.) Glucose (5 mg/ml) was added by replacing the nutrient-deficient buffer with glucose-containing buffer. Curve labeled KNRK conditioned medium: NRK cells were equilibrated for 2 h in buffer or complete Waymouth's medium or Waymouth's medium containing 10% fetal bovine serum, then exposed to the like medium, which had been incubated with KNRK cells for 2 h. Percent ruffled cells was scored as described in Materials and Methods and in Results. Each curve is the composite of three or more separate experiments. No individual value deviated more than 10% from the curves shown.

variation could be observed and a composite drawn. No individual data point varied from the composite by >10%. This method was used to derive the curves in Figs. 2, 3, and 5.

The addition of 25 μg/ml of insulin or 5 mg/ml of glucose produced ruffles on 80–100% of cells (Fig. 2). This ruffling began to decline within a few minutes and had returned to a level of 5–20% of cells within an hour even in the continued presence of the insulin or glucose. These data demonstrate that, in NRK cells, the on and off kinetics of glucose-induced ruffling are similar to those of insulin. The magnitude of the response to insulin was dose dependent (Fig. 3), with doses of ≥0.25 μg/ml producing a maximum response. Ruffling onset kinetics, as examined by comparison of the time from addition of insulin to the 30%-ruffled time point on each curve (Fig. 3 inset), show an apparent first order dependence on insulin concentration in this dose range. Off-regulation kinetics are more difficult to determine from these data, but it is clear that off-regulation does not require saturation of the ruffling response and occurs at roughly the same time over a large dose range. Transient ruffling responses following similar time courses were observed using EGF, fibroblast growth factor, and vitamins (data not shown).

Independent Off-Regulation of Insulin and Glucose-induced Ruffling

NRK cells treated with insulin, as in Fig. 2, were resistant to further ruffling if treated with another dose of insulin (Fig. 4 *a*). Insulin-resistant cells still could respond fully to glucose (Fig. 4 *b*). Similarly, cells treated with (and therefore refractory to) 5 mg/ml glucose responded fully to insulin (Fig. 4 *c*). Thus, although untransformed NRK cells are fully capable of ruffling, their ruffling is largely a response to external stimulation, and this response is limited by specific cellular off-regulatory mechanisms.

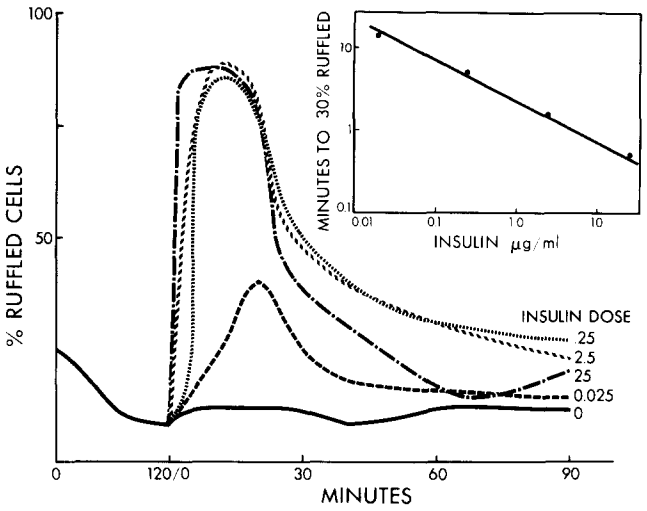

Figure 3. Percentage of NRK cells ruffled after exposure to various concentrations of insulin as a function of time. As in Fig. 2, but cells were treated with 25, 2.5, 0.25, or 0.025 μg/ml of insulin or with the same volume of hormone-free carrier (0 μg/ml), as indicated. Each curve is a composite of two separate experiments. No individual value deviated by >10% from the curves shown. For each composite curve, the time to 30% ruffled was read directly from the graph and plotted against concentration (inset). The linear relationship obtained is consistent with a first order dependence of onset kinetics on insulin concentration.

Normal Insulin and Glucose Ruffling Responses and Cellular Off-Regulation in Quiescent KNRK Cells

In contrast to NRK cells, nearly 100% of sparsely cultured KNRK cells are abundantly ruffled in complete medium. Although untransformed NRK cells became relatively quiescent in pH 7.4 buffer, KNRK cells continued to ruffle even after several hours in buffer at pH 7.4 (Figs. 1*f* and 5). However, if the pH of the equilibrating medium was maintained at 8.4, KNRK cells ceased ruffling almost entirely after 3–6 h (Figs. 1*g* and 5). This change in pH did not interfere with the intrinsic ability of cells to ruffle. The quiescent KNRK cells ruffled at pH 8.4 after the addition of insulin or glucose (Figs. 1*h* and 5) with kinetic parameters (including off-regulation) similar to those of NRK cells exposed to insulin or glucose at pH 7.4 (or 8.4, not shown). These data demonstrate that KNRK cells do require a stimulatory factor to ruffle and that ruffling responses to insulin and glucose are present and are off-regulated as in untransformed NRK cells. Thus, KNRK cells appeared to secrete their own pH-sensitive, ruffle-inducing agent(s).

Unregulated Ruffle-inducing Activity Secreted by KNRK Cells

The presence of buffer-soluble ruffle-inducing activity was demonstrated directly by its effect on untransformed NRK cells. Buffer, or complete Waymouth's medium, or Waymouth's medium containing 10% fetal bovine serum, was conditioned by preincubation on a monolayer of KNRK cells for ≥2. The conditioned medium was then placed on untransformed NRK cells which had been equilibrated in the like unconditioned medium for 2 h. Nearly 100% of the NRK cells ruffled within 1–5 min, and ruffling continued for at least 20 h (Fig. 2). If KNRK conditioned medium was premixed with antiserum to TGF-α, the ruffling response was

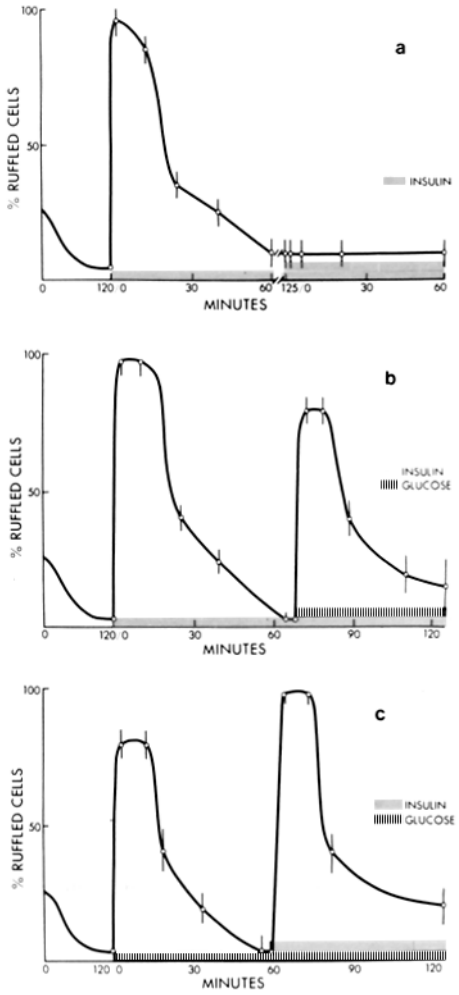

Figure 4. Response to second ruffling agent. NRK cells were treated with insulin or glucose as described in Fig. 2. After ruffling subsided, in the continued presence of the first agent, cells were challenged a second time. (*a*) Insulin as first challenge, insulin as second challenge; (*b*) insulin as first challenge, glucose as second challenge; (*c*) glucose as first challenge, insulin as second challenge. Error bars indicate area-to-area range within a count.

reduced by at least 75%. KNRK cells made quiescent by incubation at pH 8.4, then equilibrated for a few minutes at pH 7.4, also ruffled if treated with pH 7.4 KNRK conditioned medium. In contrast, NRK target cells did not ruffle if Waymouth's medium, with or without serum, was conditioned on a dense monolayer of untransformed NRK cells. (Buffer conditioned on untransformed NRK cells produced a small, transient response. This was probably due to leakage of glucose, amino acids, and other nutrients from the NRK monolayer into the nutrient-free buffer). These data demonstrate that continuous ruffling of KNRK cells is not due to a loss of cellular off-regulatory machinery or to the acquisition of an intrinsic ruffling capacity that does not require stimulation. Rather, KNRK cells secrete an autostimulatory ruffle-inducing activity which is not off-regulated by either transformed or untransformed NRK cells and which appears to include TGF-α as an active component.

Discussion

Real-time visual observation of changes in the percentage of

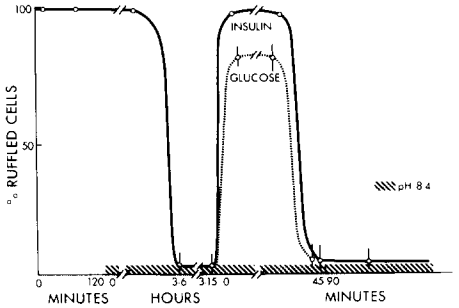

Figure 5. pH-dependent loss of autonomous KNRK ruffling and percentage of quiescent KNRK cells ruffling after exposure to insulin or glucose. KNRK cells were equilibrated for 2 h in buffer under 5% CO_2 atmosphere, then moved to an ungassed atmosphere, which allowed the buffer to drift to pH 8.4. Autonomous ruffling ceased after 3–6 h. After 3–15 h at pH 8.4, cells were treated with 25 μg/ml insulin or 5 mg/ml glucose, as in Fig. 2, and percent ruffling cells was determined. Each curve is the composite of three or more separate experiments; there was <10% variation at any value.

live cells ruffling provided a reliable measure of the kinetics of an early cellular response to growth factors and nutrients. There are potential difficulties with this method, because some background ruffling is present normally in cultured cells, and cells ruffle in response to numerous perturbations. In the present study, the background ruffling was reduced to an acceptable minimum by preincubation of the cells in nutrient deficient buffer. The validity of the individual ruffling responses was demonstrated by several observations. Blank carrier did not produce ruffling (Fig. 3). After a saturating dose, a second dose of the same agent did not produce ruffling, although a different agent did. Finally, the magnitude and onset kinetics of the insulin response were dose dependent.

This method was used to demonstrate the striking ability of cells to off-regulate their ruffling responses to all of the normally occurring (as distinct from transforming) growth factors and nutrients tested. This off-regulation was not merely a saturation phenomenon, since it occurred even at a dose of insulin that produced a partial response. Nor was it due to a paralysis of the ruffling mechanism, since cells refractory to one factor could still ruffle if treated with another factor. Down-regulation and up-regulation of specific surface receptors is a well-known mechanism by which cells modulate their responsiveness to a particular polypeptide hormone as a function of the ambient concentration of that hormone. Down-regulation of the insulin receptor occurs rapidly after exposure to insulin (2) and thus may play a role in the off-regulation observed here. Subsequent observations in this laboratory (manuscript in preparation) indicate that other mechanisms may also be involved. Furthermore, glucose and vitamins, which produced similar off-regulation kinetics, do not have cell surface receptors analogous to those of the polypeptide hormone receptor family. But, whatever the mechanism, these cells exhibited both the capacity to respond to exogenous factors and the ability to limit those responses specifically.

In contrast to NRK cells, KNRK cells ruffle intensely and continuously. In these and numerous other cells, enhanced ruffling appears to be a legitimate morphological sign of the transformed phenotype (1, 19, 21). It was interesting to observe that the transformed morphology of the individual

KNRK cells was largely reversed after a few hours at pH 8.4. However, since both the high pH and the nutrient deficiency were unphysiological conditions, no other parameters, such as growth rate or intercellular interactions, could be correlated with the altered morphology. In this study, increased pH was used merely to eliminate the endogenous ruffling of KNRK cells so that the kinetics of their insulin and glucose responses could be observed. Since the insulin and glucose-induced ruffling of KNRK cells at pH 8.4 resembled the responses of NRK cells at physiological pH (or, in fact, of NRK cells at pH 8.4), the conditions were considered to be valid for the questions asked. Those questions were, do KNRK cells require a stimulus to ruffle, and are KNRK cells capable of off-regulation? In introductory paragraphs of this paper we suggested four alternative hypotheses to explain the enhanced and apparently unregulated ruffling of KNRK cells. The data presented clearly reject hypotheses *a–c* and support hypothesis *d*: KNRK cells secrete their own ruffle-inducing factor or factors which are not off-regulated by either transformed or untransformed NRK cells.

The molecular composition of the KNRK tumor ruffling agent(s) (TRA) has not yet been determined. Other known ruffle-inducing factors fall into two broad classes: peptide mitogens and nutrients. It seems unlikely that the TRA is a nutrient, particularly since it appears to be sensitive to increased pH. The autostimulatory nature of the TRA resembles the previously described "autocrine" growth stimulation (29) of transforming growth factors (TGFs) (30, 31). Furthermore, both TRA and TGFs induce cellular responses that are similar in kind (not duration) to the cellular responses to exogenous growth factors (20). One of the two major TGFs, TGF-α has EGF-like biological activity and binds to the EGF receptor (3). The other, TGF-β enhances the activity of either TGF-α or EGF, reversibly producing a transformed phenotype in untransformed target cells, although it does not have growth factor activity itself (3). These TGFs are secreted by KNRK cells (20). Platelet-derived growth factor is also secreted by Kirsten murine sarcoma virus–transformed cells (5). Both EGF (reference 7, 8, 14, and 15, and this study) and platelet-derived growth factor (28) induce ruffling in cells, although transiently. Thus it seems likely that some combination of these known factors may be part or all of the TRA described here. In this study, an antiserum against TGF-α largely inhibited TRA-induced ruffling, which strongly suggests that TGF-α is an active component of the TRA. The accompanying paper describes the roles of TGF-β and TGF-α in the induction and maintenance of ruffling.

Whatever the composition of the TRA, it appears to be sufficient to induce the continuous ruffling seen on KNRK cells. Thus, as with many other cellular activities, ruffling in these transformed cells has become an autonomously induced behavior controlled by secreted factors (autocrines [29]) instead of a limited response to environmental factors. Ruffling is a poorly understood phenomenon. But the following evidence suggests the possibility that ruffling relates to rapid alteration of plasma membrane function, especially augmentation of nutrient transport. Several types of cellular events are elicited by growth factors: ruffling (this study and referenced above), enhanced membrane transport and/or increased cell surface transport molecules (9, 12, 13, 17, 23, 24, 27, 35), enhanced rates of both endo- and exocytosis (14, 34, 35), and accumulation of clathrin-coated membrane at the cell surface (8). All of these events exhibit similar acute kinetics, suggesting that ruffling may be a manifestation of a membrane relocation that inserts transport molecules into the cell surface. Increased hexose transport has also been associated with transformation by RNA viruses (4, 10, 15, 22, 26, 32, 33). Thus, transformation-associated ruffling may be homologous to ruffling caused by growth factors, and both may be related to increased rates of nutrient transport. TRA, which deregulates ruffling, may correspondingly deregulate nutrient transport.

In the present study, comparison of ruffling kinetics has given us a way to observe similarities and differences between early cellular responses to nontransforming growth factors and the TRA secreted by KNRK cells. Both growth factors and the TRA rapidly caused ruffling on most target cells, whether transformed or untransformed. The key difference was the failure of target cells to turn off their response to TRA. It is clear that the transforming property of a factor secreted by tumor cells must be more than the similarity of that factor to nontransforming growth factors. It must, in fact, be the difference in the response it elicits in cells. This study demonstrates that, for the ruffling response, a normally well-regulated function, the most obvious difference between TRA and a variety of nontransforming growth factors or nutrients is the escape from cellular off-regulation.

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