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REGULATION OF SIGNALLING BY RECEPTOR TYROSINE KINASES IN HeLa CELLS INVOLVES THE q-BASE

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The nutrition factor queuine (q; the q-base) is a modulator of mammalian cell proliferation: it can be inhibitory or stimulatory, depending on the metabolic state of the cell. The mechanism underlying this growth-modulating activity was investigated. It was found that the q-base acts antagonistic to epidermal growth factor (EGF) but synergistic with platelet-derived growth factor (PDGF) in HeLa cells. Binding of either growth factor to its receptor resulted in the activation of distinct cellular kinases. The activities of these kinases were profoundly affected by q. The results suggest that the q-base is involved in the homologous regulation of signalling by receptor tyrosine kinases.

Recently obtained results suggested that the 7-deazaguanine-derivative, queuine, might be involved in the regulation of signal transduction pathways in mammalian cells (1, 2, 3). Queuine is synthesized by bacteria and occurs in the anticodon of specific tRNAs (reviewed in 4, 5, 6). Mammals -like other eucaryotes- cannot synthesize queuine, but obtain it from nutrition as a degradation product of bacterial tRNA. In the intact mammalian organism queuine is ubiquitously present in two states: as a free base (the q-base, abbreviated as q) outside and inside the cell, and in a tRNA-bound state within the cell as modified nucleoside queuosine (Q). Cultured cells can become q-deficient (6), especially when grown in horse serum or in the presence of low amounts of fetal serum, as is often the case when growth experiments or stimulation experiments with growth factors are performed. The q-base acts as growth promotor or as growth inhibitor in cultured mammalian cells, depending on their aerobic or glycolytic metabolic predisposition: in serum-supplemented medium it stimulates proliferation of aerobically grown but inhibits proliferation of hypoxically grown HeLa cells (7). The q-base is essential for this type of adaptive regulation of proliferation in HeLa cells. In an attempt to clearify the mechanism underlying this modulating activity of the q-base, I have analyzed the effect of q on the mitogenic signal produced by the polypeptide growth factors EGF and PDGF, two ligands for receptors with intrinsic tyrosine kinase activity (reviewed in 8, 9). The results presented here suggest that the q-base acts antagonistic to EGF, but synergistic with PDGF and -because of its ubiquitous presence- might be important in the regulation of signalling by these two growth factors *in vivo*.

Materials and Methods

Minimum essential medium (MEM) was from Biochrom KG, Germany. Horse serum was from Boehringer Mannheim, Germany. Human recombinant EGF and PDGF were from Biomol,

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Germany. [γ^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham, Germany. HeLa-S3 cells were obtained from the American Type Culture Collection.

HeLa cells were routinely grown in MEM supplemented with 10% horse serum in an atmosphere of 95% air and 5% CO_2. For proliferation assays, they were plated in 5 cm culture dishes containing 4 ml MEM supplemented with 5% horse serum at a density of 5000 cells/cm^2. After inoculation for two to three days, factors were added without exchanging the medium in the following order: chemically synthesized q-base (final concentration 300 nM), PDGF (final concentration 0.8 nM), and/or EGF (final concentration 1 nM or 10 to 45 nM). Cells were further incubated, and the total cell number per culture dish was determined 24 and 48 hours later, by detaching the cells with trypsin and counting microscopically or automatically using a coulter counter (CASY 1, Schärfe System, Germany).

Cellular kinase activities were analyzed as described for the MAP kinases (10). HeLa cells were grown in 10 cm culture dishes in 10 ml MEM/10% horse serum, starting with 1 x 10^6 cells. The medium was replaced with serum-free MEM after two days, and the cells were incubated for another two days. Cells were then washed twice with PBS, were scraped into PBS, and collected by centrifugation. Cells from 2 dishes were pooled (approximately 1.5 x 10^7 cells) and resuspended in 4 ml PBS. The cell suspension was then distributed to 1.5 ml Eppendorf caps (200 µl/cap) and stimulated with EGF (5 nM), or EGF and PDGF (0.8 nM), in the absence or presence of the q-base (2 µM). PDGF was added before EGF, and the q-base before growth factors. After 2, 5, 10, and 30 min respectively, cells were briefly spun down in a microfuge. The supernatant was discarded, and the cells were placed on ice. The cells were lysed in 100 µl ice-cold 20 mM Hepes, pH 7.4, 0.5% Triton X-100, 25 mM 2-glycerophosphate, 5 mM 2-mercaptoethanol, and 2 µg/ml each of pepstatin, leupeptin, aprotinin, α_2-macroglobulin. Nuclei were removed by centrifugation, and the supernatant was used for *in vitro* phosphorylation. Assays were performed in a final volume of 20 µl of the above mentioned lysis buffer containing 10 µg of protein, 10 mM $MgCl_2$, and 5 µCi [γ^{32}P]ATP. After 15 min at room temperature, the reaction was stopped by the addition of electrophoresis sample buffer and boiling for 2 minutes. Samples were applied to 15% or 10% PAGE. The gels were stained with Coomassie Brilliant Blue, dried and exposed to Kodak Xomat films. Gels were then rehydrated and treated with 1 M NaOH at 55°C for 20 min. After washing in an extensive volume of 7.5% acetic acid/5% methanol, the gels were dried and exposed again.

Results

HeLa cells were cultivated in medium supplemented with horse serum that contains, if at all, very low amounts of q (1 nM or less) (6). These cells cannot adapt their proliferation to changes in oxygen availability (7). However, when the cells are supplied with 300 nM chemically synthesized q-base they grow faster under aerobic but slower under hypoxic conditions than the q-deficient cells, suggesting that the q-base acts synergistic with serum in oxygenated cells but antagonistic in glycolytic cells. When HeLa cells were grown in the presence of only 5% horse serum for two to three days, addition of the q-base no longer stimulated proliferation (Figure 1A), indicating that q is not a growth factor itself but acts in conjunction with serum factors that can become depleted after prolonged culture. These culture conditions were used to study the effect of q on the mitogenic signal initiated by polypeptide growth factors. The proliferation of HeLa cells increased 30 to 50% within 48 hours when EGF was added to a final concentration of 1 nM (Figure 1A). The EGF-induced increase in proliferation was completely abrogated when EGF was added at this concentration together with the q-base (300 nM) (Figure 1A). This shows that EGF at low doses is not mitogenic for HeLa cells in the presence of the q-base (reflecting the *in vivo* situation). The inhibitory effect of q on the EGF-supported proliferation was overcome by adding EGF at higher concentrations (10 nM or more).

Treatment of HeLa cells with PDGF (0.8 nM) under the conditions described above had no effect on proliferation (Figure 1B), indicating that PDGF alone is not mitogenic for HeLa cells. Also, a simultaneous addition of PDGF and 1 nM EGF did not produce any significant increase in proliferation (Figure 1B),suggesting that PDGF also prevents the mitogenic effect of low doses of

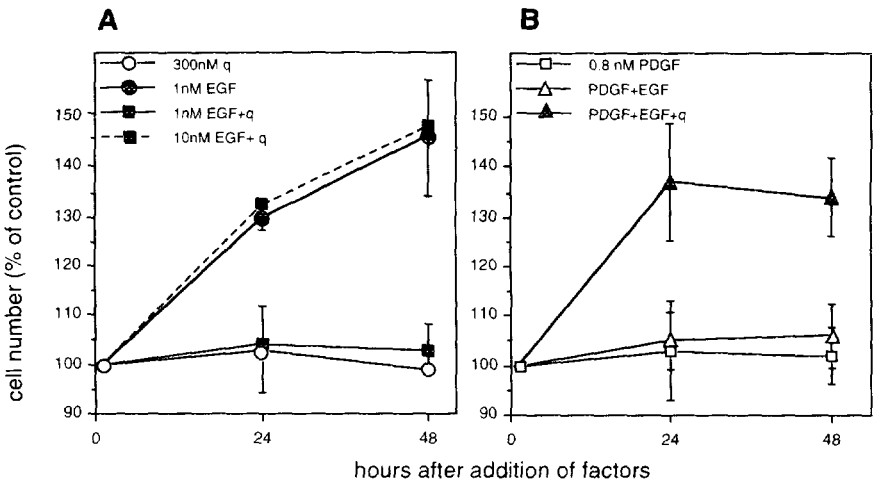

Fig. 1. Proliferation of HeLa cells in the presence of polypeptide growth factors. The cells were precultivated for 2 to 3 days, followed by addition of growth factors and q as indicated. The concentration of the q-base was 300 nM. In panel B, EGF was added to a concentration of 1 nM. Values are means ± SDM from four independent experiments and are expressed as percent increase in cell number over control values (no addition of factors = 100%).

EGF. However, proliferation increased 25 to 45% when the q-base was added in combination with PDGF and EGF (Figure 1B).

Evidence was provided recently that the q-base influences signalling pathways initiated by EGF and , probably, other growth factors by influencing kinase activitites (2, 3). Therefore, protein phosphorylation was investigated after treatment of intact HeLa cells with EGF alone or with PDGF and EGF in the absence or in the presence of the q-base. Lysates were prepared at various times after stimulation and were incubated with [γ^{32}P]ATP. Because of the presence of phosphatase inhibitors this assay allows the analysis of endogeneous kinase activities as described for instance for the MAP kinases (10). Treatment of HeLa cells with EGF induced the transient activation of (a) kinase(s) that caused phosphorylation of proteins with M_r 105 and 58 kDa (pp105 and pp58 in Figure 2A) This EGF-induced phosphorylation was prevented by PDGF, confirming that PDGF abrogates signalling by the EGF receptor. Only in the presence of the q-base, PDGF caused activation of (a) different kinase(s) responsible for the phosphorylation *in vitro* of proteins with M_r 110 and 44/45 kDa (pp110 and pp44/45 in Figure 2A). This reflects a synergistic effect of q on signalling by the PDGF receptor. Phosphate incorporated into pp44/45 was removed by alkali-treatment, suggesting phosphorylation on serine residues (Figure 2B). Phosphate in pp110 was alkali-stable, suggesting phosphorylation on others than serine residues. A protein with M_r 16 kDa was phosphorylated in lysates derived from EGF-treated cells (pp 16 in Figure 1A). The kinase activity responsible for this phosphorylation was apparently suppressed when q was present during EGF-treatment, suggesting an antagonistic effect on the signal initiated by EGF in this case. However, it should be noted that the q-base enhanced an EGF-induced kinase activity that causes phosphorylation of pp105. This becomes most obvious after alkali-tratment (Figure 2B), suggesting phosphorylation on threonine and/or tyrosine residues. Thus, unlike PDGF, q does not prevent signalling by the EGF receptor, although it inhibits EGF-supported proliferation.

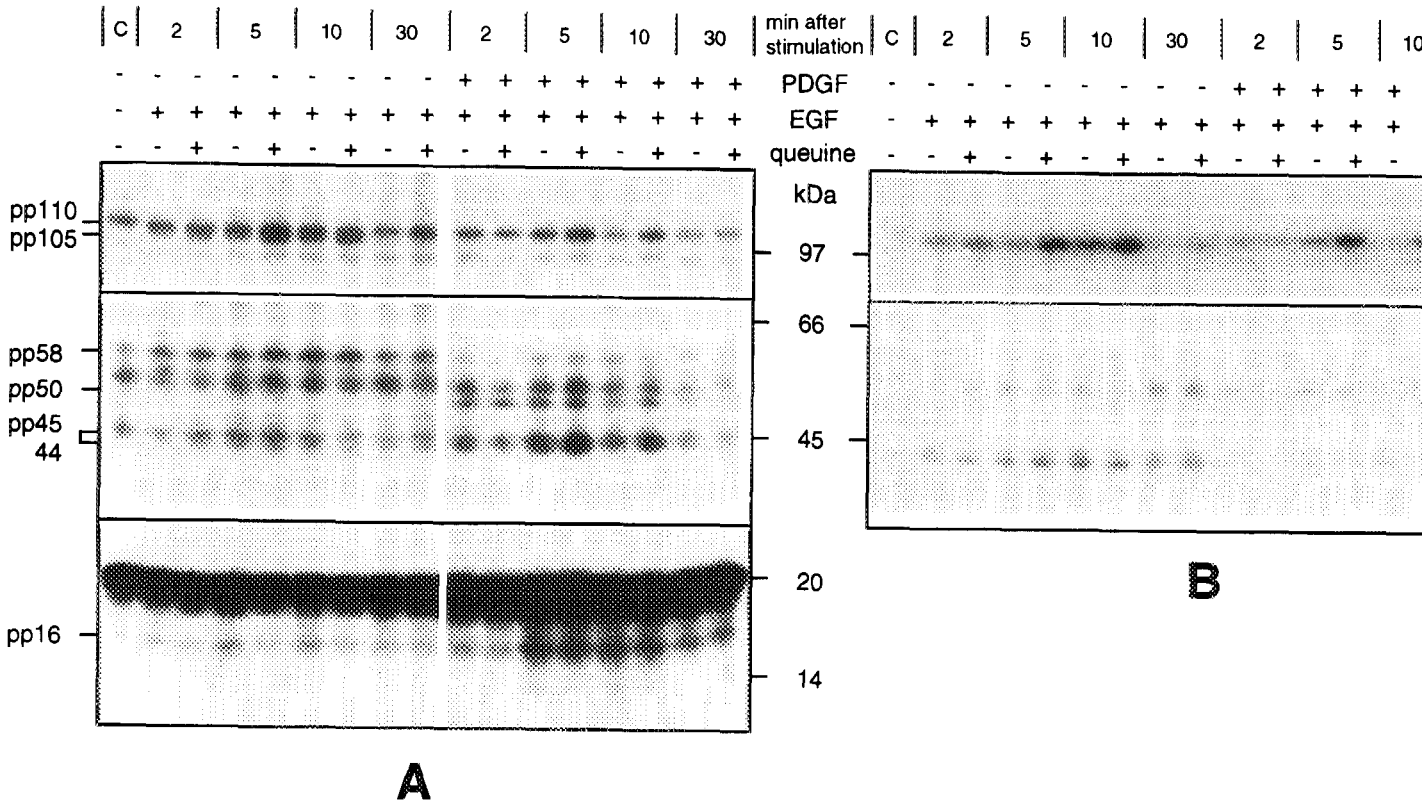

Fig. 2. Protein phosphorylation in extracts from HeLa cells treated with growth factors in the absence or presence of the q-base. Serum-starved HeLa cells were stimulated with EGF alone (5 nM), or with PDGF (0.8 nM) and EGF, in the absence or presence of the q-base (2 μM). Protein extracts were prepared at the noted times after stimulation and were incubated with [γ^{32}P]ATP for 10 min (C = control before stimulation). Samples were applied to 10% (upper 2 panels) or 15% PAGE (lower panel in A). The gels were dried and exposed for different periods (from top to bottom: 30 min at room temperature, 8 hours at -70°C with an intensifying screen, and over night at -70°C with an intensifying screen). (B) The same 10% gel that is shown in A was rehydrated after exposure, incubated in 1 M NaOH at 55°C for 20 min, washed in destaining solution and was exposed again.

Discussion

Peptide growth factors are components of a complex biological signalling language, providing the basis for cell-cell communication in a multicellular organism. Cellular signalling is crucial for the regulation of proliferation, differentiation, survival and metabolic homeostasis (11, 12). Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are ligands for cell surface receptors with intrinsic tyrosine kinase activity (8, 9). Linear signalling cascades from a single activated receptor to the nucleus have been elaborated recently (13, 14, 15). However, in the intact organism, peptide growth factors always act in sets and little is known about the mechanism how the cell selects and integrates signals from more than one origin. Here it is shown that one growth factor can be dominant over another: activation of the PDGF receptor abrogates the mitogenic potential of low doses of EGF. This is best explained by the observation made with fibroblasts that PDGF abolishes high affinity binding sites for EGF by inducing phosphorylation of the EGF receptor at threonine 654 (16). The K_d value for the high affinity EGF-binding sites in HeLa cells has been calculated to 0.12 nM, and that of the low affinity sites to 9.2 nM (17). Assuming that PDGF also abolishes high affinity binding sites for EGF in HeLa cells, then EGF at a concentration of 1 nM would no longer be effective in stimulating mitogenesis. This is in agreement with the observations described here. However, proliferation increased 25 to 45% when the q-base was added in combination with PDGF and EGF. Most likely, this increase is not caused by an inactivation of the PDGF receptor system by the q-base, because proliferation is not stimulated in the presence of EGF and q. Apparently, the q-base acts synergistic (or even permissiv) with PDGF in HeLa cells. This may account for the stimulatory effect of q on the proliferation of aerobically predisposed cells in serum-supplemented medium observed previously (7). The phosphorylation experiments indicate that the synergistic effect of q with PDGF is also brought about by an enhancement of the signal generated by the PDGF receptor. The q-base apparently represents a novel level at which the PDGF receptor signalling cascade can be regulated.

The mitogenic potential of low doses of EGF was abrogated also by the q-base, suggesting that this ubiquitously present factor is a physiological antagonist of EGF. The inhibitory effect of q was overcome by higher amounts of EGF. Because the q-base is found in all mammalian body fluids and organs, this raises the intriguing possibility that the presence of the q-base may provide a threshold for the mitogenic action of EGF *in vivo*. Strikingly, the q-base -unlike PDGF- did not abolish signalling by the EGF receptor: the EGF-induced phosphorylation of one protein (pp16) was prevented by the q-base, that of a second (pp58) was not affected at all, and that of pp105 was even enhanced. This suggests that the mechanism by which the q-base neutralizes the mitogenic effect of EGF is different from the PDGF-mediated mechanism. The present results rather confirm the previously made assumption that modulation of the autophosphorylation of the EGF receptor by q is the primary effect that sorts the signal in a different direction (2).

Because of its ubiquitous presence, the q-base is likely to participate in the regulation of signalling events in mammals *in vivo*. For example, EGF might not be mitogenic for some cells at low doses when q is present. Only when the amount of EGF increases (e.g. after injury, or in embryogenesis), proliferation may be induced even in the presence of q. On the other hand, PDGF may be a mitogen for some cells only in combination with q acting as a co-mitogen.

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