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Epidermal growth factor induces protein tyrosine phosphorylation and association of p190 with ras-GTP-ase activating protein in Caco-2 cells

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Abstract Epidermal growth factor (EGF) modulates several functions of human enterocytes. We report that this growth factor induces strong tyrosine phosphorylation stimulation of its receptor and several putative substrates of the receptor intrinsic kinase including c-erb B2 in proliferating human colon carcinoma cells (Caco-2). In addition EGF induces stable association of the GTP-ase activating protein of $p21^{ras}$ to the p190 protein and to a 62 mol.wt. tyrosine-phosphorylated protein. This association is probably consequent to EGF stimulation of protein tyrosine phosphorylation and could coordinate progression through cell cycle with polarity, cell–cell interactions and cell mobility.

Key words: Signal transduction; Tyrosine phosphorylation; ras-GAP, p190, p62, c-erb B2

1. Introduction

EGF stimulates proliferation of intestinal cells [1] modulates in vitro human enterocyte (Caco-2) migration [2], and maintains the integrity of the gastrointestinal mucosa [3]. In addition, gene expression of a trefoil peptide PS2, member of a growing family of proteins associated with mucosal repair and suspected to be involved in neoplasms of the gastrointestinal tract, is regulated by EGF [4]. The Caco-2 cells, derived from human colon carcinoma [5,6] contain about 6,000 EGF-R per cell unevenly distributed between the apical and basolateral membrane [7,8] and respond to EGF with growth [9].

We observe that in subconfluent, proliferating Caco-2 cells EGF strongly increases the basal level of tyrosine phosphorylation of its own receptor and several other proteins which are putative substrates of EGF-R including c-erb B2 (also called neu or HER-2). In addition EGF induces stable association of p190, a recently cloned protein [10], to ras-GAP (the p120 GTP-ase activating protein), likely through p190 tyrosine phosphorylation. p190 association with ras-GAP is increased during tyrosine kinase mediated cell proliferation and this association reduces the GTP-ase stimulatory activity of ras-GAP [11] increasing the active form of ras [12]. It has recently been observed that p190 is endowed with a GAP activity specific for rho, a ras-related protein family [13]. Therefore complex formation between ras-GAP and p190 following growth-factor stimulation offers a mechanism to couple signals mediated by $p21^{ras}$ and rho GTP-ases [13]. Since rho proteins regulate actin cytoskeleton [14,15] and cell polarity [16] whereas $p21^{ras}$ transduces signals for mitogenesis, our data suggest that the EGF-dependent ras-GAP–p190 association coordinates progression through the cell cycle with polarity, cell–cell interactions and motility of intestinal cells. In addition, EGF stimulates tyrosine phosphorylation and/or ras-GAP association of a 62 kDa protein, very likely to be the p62 protein [17].

2. Materials and methods

2.1. Materials

Human recombinant EGF was from Boehringer (Mannheim, Germany). Mouse monoclonal anti EGF-R antibodies, mouse monoclonal Ig2bk anti-P-tyrosine antibodies (clone 4G10), polyclonal rabbit anti-GAP antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Polyclonal rabbit anti-c-erbB-2 antibodies (M6) were a generous gift from Dr. P.P. Di Fiore (Molecular Biology Laboratory of NCI, NIH, Bethesda, MD, USA). Anti p-190 rabbit antisera were a gift from Dr. J. Settleman (MGH Cancer Center, Charlestown, MA, USA). Anti-mouse and anti-rat IgG, alkaline phosphatase conjugates were from Promega (Madison, WI, USA). Reagents for cell culture media including foetal calf serum were from Gibco (Gaithersburg, MD, USA).

2.2. Cell culture and EGF treatment

Caco-2 cells were a generous gift from Dr. G. Tritto (Cattedra di Gastroenterologia, Università di Napoli "Federico II", Napoli, Italy). They were routinely grown in 10% CO_2 in air atmosphere using Dulbecco modified Eagle's medium (DMEM) supplemented with Phenol red, L-glutamine (2 mM), penicillin (100 $U \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), 10 mM HEPES and 10% foetal calf serum. Subconfluent cells grown in 100 or 145 mm Petri dishes were kept in the same medium lacking foetal calf serum and added to 25 $\mu g \cdot ml^{-1}$ transferrin, 25 $ng \cdot ml^{-1}$ insulin and 20 mM sodium selenite for 18 h before the EGF treatment. Cells were then incubated with or without EGF at indicated concentrations for 15 min.

2.3. Analysis of tyrosine phosphorylation and immunopurification by anti-P-tyrosine antibodies of total proteins from EGF-treated CaCo-2 cells

Cells were washed with ice-cold phosphate-buffered saline (PBS), pH 7.4, scraped and added to 1 ml of lysis buffer: 50 mM Tris-HCl, EDTA 4 mM, 150 mM KCl, 1 mM PMSF, 1 $\mu g \cdot ml^{-1}$ of each antipain, leupeptin and pepstatin, 10 $\mu g \cdot ml^{-1}$ of soy bean trypsin inhibitor, 1 mM sodium orthovanadate and 1% Triton X-100, pH 7.4. The lysate was diluted with lysis buffer to a final concentration of 2.5 $mg \cdot ml^{-1}$ of proteins. Hundred μg of proteins were mixed with Laemmli sample buffer and submitted to SDS-PAGE followed by blot with anti-P-tyrosine antibodies. In the immunopurification experiments by anti-P-

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Abbreviations: BIS, *N,N'* methylene-bis-acrylamide; EGF, epidermal growth factor; EGF-R, EGF-receptor; SDS, sodium dodecyl-sulphate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenyl-methyl-sulfonyl-fluoride; ras-GAP, ras-GTP-ase activating protein; TEMED, *N,N,N',N'*-tetramethyl-ethylene-diamine; Tris, Tris [hydroximethyl]-aminomethane.

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tyrosine antibodies 1 ml of cell lysate was diluted with 1 ml of lysis buffer lacking Triton X-100 and incubated overnight with 100 μl of anti-P-tyrosine antibodies linked to agarose. The suspension was centrifuged and the beads were washed with lysis buffer containing 0.1% Triton X-100 (washing buffer) and proteins eluted by incubating the beads with 100 μl of Laemmli sample buffer for 3 min at 100°C. The eluate was submitted to SDS-PAGE and blotted with either anti-P-tyrosine or anti-EGF-R antibodies.

2.4. Immunopurification of EGF-R

One ml lysate was diluted with lysis buffer to a final concentration of 2.5 mg·ml^{-1} of proteins and incubated for 1 h with 5 μg of mouse IgG and 40 μl of protein G-sepharose (50% suspension). The samples were centrifuged and supernatants incubated overnight with 5 μg·ml^{-1} of either control or anti EGF-R antibodies. Each sample was added to 120 μl of protein G-Sepharose (50% suspension) and incubated for an additional hour. The samples were centrifuged, the pellets were washed with washing buffer and proteins eluted by boiling in 150 μl of Laemmli sample buffer. The eluates were submitted to electrophoresis and blotted with either anti-P-tyrosine or anti-EGF-R antibodies.

2.5. Immunopurification of c-erb B2

Cells were added to 1.2 ml of lysis buffer containing 1% aprotinin in addition to the other components. The lysate was diluted with lysis buffer to a final concentration of 4.5 mg·ml^{-1} of proteins. Two 500 μl fractions were incubated for 1 h with 5 μg of either rabbit control or M6 anti-c-erb B2 polyclonal antibodies. Then 70 μl of protein G-Sepharose suspension (50% in lysis buffer, v/v) was added to each sample and the incubation continued for 45 min. The pellets were collected by centrifugation, washed with 1 ml of lysis buffer, then eluted by boiling in 70 μl of Laemmli sample buffer. The eluates were resolved on SDS-PAGE and blotted with either anti-c-erb B2 or anti-P-tyrosine antibodies.

2.6. Immunopurification of ras-GAP and ras-GAP-associated proteins

The lysate was diluted with lysis buffer to a final protein concentration of about 1.5 mg·ml^{-1} and incubated for 1 h with 1 μg·ml^{-1} of rabbit IgG and 100 μl of protein G-Sepharose suspension. The sample was centrifuged and the supernatant incubated with either 8 μg·ml^{-1} of rabbit IgG or 8 μg·ml^{-1} rabbit polyclonal anti-ras-GAP antibodies overnight at 4°C. The samples were then added to 70 μl of protein G-sepharose and incubated for additional 90 min. They were centrifuged and pellets washed with washing buffer and eluted by boiling in 120 μl of Laemmli sample buffer. Each eluate was submitted to electrophoresis and blotted with either anti-P-tyrosine or anti-ras-GAP or anti-p190 antibodies.

To immunoprecipitate the ras-GAP associated p190, 1 ml of lysate prepared and diluted as described above was incubated for 1 h with 1 μg·ml^{-1} of rabbit IgG and 80 μl of protein G-Sepharose suspension. The sample was centrifuged and the supernatant incubated overnight with either 2 μg·ml^{-1} of rabbit IgG or 2 μg·ml^{-1} of rabbit polyclonal anti-p190 antibodies. The samples were then added to 60 μl of protein G-sepharose and incubated for further 60 min. They were centrifuged, washed and eluted by boiling in 120 μl of Laemmli sample buffer. The eluates were submitted to SDS-PAGE and blotted with either anti-P-tyrosine or anti-p190 antibodies.

2.7. Electrophoresis and immunoblotting

Sixty μl samples were submitted to SDS-PAGE (7.5% acrylamide, acrylamide/BIS ratio 37,5:1) using 10 × 15 cm gels. After separation, proteins were electrophoretically blotted onto nitrocellulose filters (S&S BA 85) at 25 V overnight at room temperature by using a transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol. The nitrocellulose filters were soaked in 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0 (TBST buffer) containing 3% BSA (blocking solution) and incubated for about 2 h. They were then incubated for a further 2–3 h, with either anti-P-tyrosine or anti-EGF-R antibodies (1 μg·ml^{-1} in blocking solution) or anti-ras-GAP (1:1000 dilution) or anti-p190 antibodies (1:500 dilution) of M6 anti-c-erb B2 antibodies (1:1000 dilution); thereafter pellets were collected and washed for 10 min with TBST buffer. The filters treated with anti-P-tyrosine or anti-EGF-R antibodies were incubated with alkaline phosphatase-linked anti-mouse IgG antibodies (1:4000 dilution in TBST buffer) and the filters treated with anti-ras-GAP or anti-p190 or anti-c-erb B2 antibodies were incubated with alkaline phosphatase-linked anti-rabbit IgG antibodies (1:7500 dilution in TBST buffer) for 45 min at room temperature. Finally, filters were washed again and protein–antibody complexes revealed according to the instructions of manufacturer.

3. Results

3.1. Dependence of total protein tyrosine phosphorylation on EGF concentration

Caco-2 cells untreated or treated for 15 min with different concentrations (from 1 to 100 ng·ml^{-1} of medium) of EGF were lysed; the lysates were submitted to SDS-PAGE and blotted with anti-P-tyrosine antibody. In the absence of exogenous EGF (0 lane) several tyrosine phosphorylated proteins were detected (Fig. 1). EGF treatment stimulated tyrosine phosphorylation of some of these proteins. The strongest stimulation was observed at 100 ng·ml^{-1} (16 nM) EGF. In other experiments optimal stimulation was detected at 200 ng·ml^{-1} EGF. The most strongly stimulated protein is one which like the EGF-R migrated at 170.000 mol.wt. Minor although significant stimulation of phosphorylation of a 185.000 mol.wt. protein was detected (Fig. 1) at the highest EGF concentration. Its molecular weight corresponded to c-erb B2 which is also a receptor-associated tyrosine kinase.

3.2. Purification of proteins by anti-P-tyrosine antibody coupled to agarose followed by blots with either anti-P-tyrosine or anti-EGF-R antibodies

Lysates from subconfluent Caco-2 cells untreated or treated with EGF were incubated with beads of anti-P-tyrosine-aga-

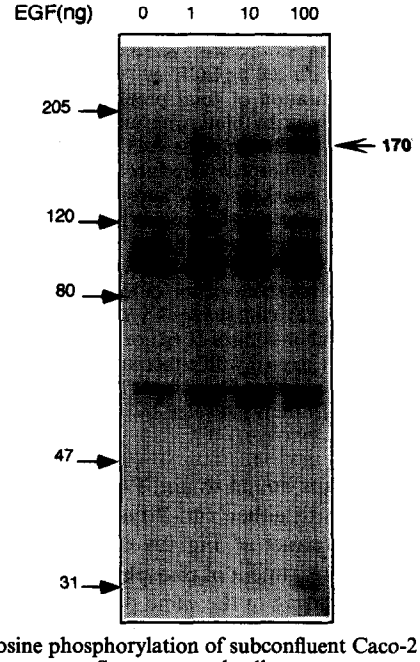

Fig. 1. Tyrosine phosphorylation of subconfluent Caco-2 cell proteins: EGF dose–response. Serum-starved cells were not treated (lane 0) or treated with different concentrations (1, 10 and 100 ng·ml^{-1}) of EGF for 15 min at 37°C, then lysed. Total cellular proteins (100 μg) were separated on SDS-PAGE (7.5% polyacrylamide) and blotted with anti-P-tyrosine antibodies. Mol.wt. markers (×10^{-3}) are indicated by arrows at the left. The position of the 170×10^{-3} mol.wt. tyrosine-phosporylated protein is indicated by the arrow at the right.

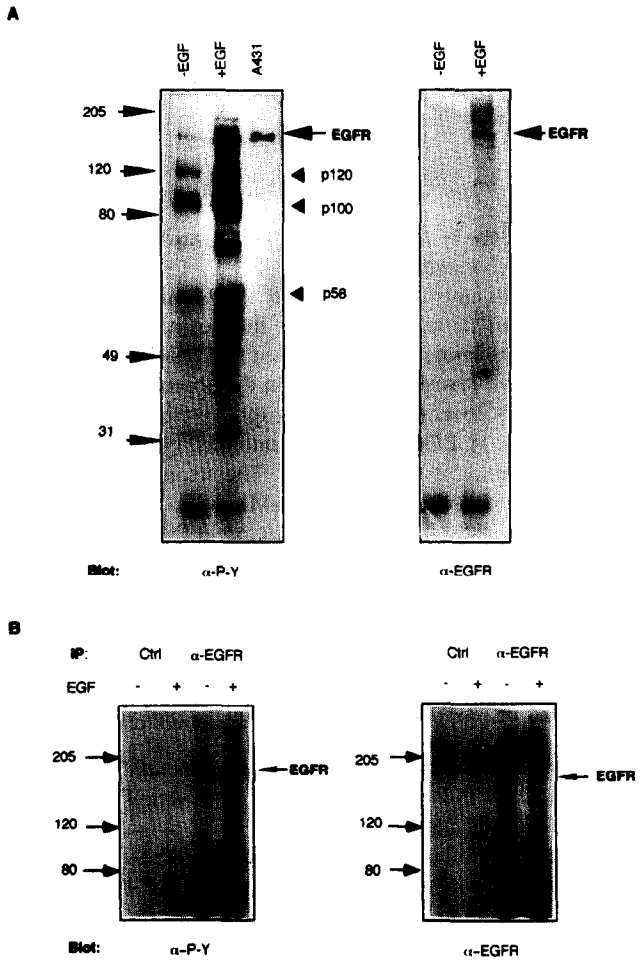

Fig. 2. Effect of EGF on tyrosine phosphorylation of total proteins and EGF-R in subconfluent Caco-2 cells. Lysates from serum-starved cells not treated or treated with 200 ng EGF·ml^{-1} for 15 min at 37°C were used. (A) Immunopurification of total proteins (2.5 mg) by anti-P-tyrosine antibody followed by blots with anti-P-tyrosine (α-p-Y) or anti-EGF-R (α-EGFR) antibodies. The A431 lane was loaded with a preparation of membrane phosphorylated protein from A431 cells stimulated by EGF. Mol.wt. markers (×10^{-3}) are indicated at the left. The numbers at the right of the anti-P-tyrosine blot indicate the mol.wt. (×10^{-3}) of the more represented putative EGF-R substrates. At the right of the anti-EGF-R blot the position of EGF-R is indicated. (B) Analysis of tyrosine phosphorylation of EGF-R in the absence and presence of EGF. Lysates containing 3.5 mg of protein were immunoprecipitated by either control (Ctrl) or anti-EGF-R (α-EGFR) antibodies and proteins blotted with either anti-P-tyrosine (α-P-Y) or anti-EGF-R antibodies. Mol.wt. markers (×10^{-3}) are indicated at the left. The position of EGF-R is indicated by the arrows at the right.

rose antibody. Proteins bound to anti-P-tyrosine-agarose were eluted and blotted with either anti-P-tyrosine or anti EGF-R antibodies. The left panel of Fig. 2A depicts the impressive stimulation by EGF treatment of phosphorylation of a 170.000 mol.wt. protein migrating in the same position of the EGF-R from A 431 cells which was used as a reference protein (A 431 lane). This co-migration suggested that the 170.000 mol.wt. protein observed in EGF-treated cells was the EGF-R. This was supported by the finding that using lysate of EGF-treated cells the 170.000 mol.wt. protein was recognized in blot by anti-EGF-R antibody (right panel of Fig. 2A). The left panel of Fig. 2A shows that in addition to the 170.000 mol.wt. protein those migrating at about 100.000 and 56–60.000 mol.wt. are also heavily tyrosine-phosphorylated in EGF-treated Caco-2 cells. Furthermore, above the EGF-R it is possible to detect stimulation of the 185.000 mol.wt. protein, the putative c-erb B2.

3.3. Tyrosine phosphorylation of EGF-R

To furnish additional and conclusive evidence that the 170.000 mol.wt. tyrosine-phosphorylated protein observed in EGF-treated cells is indeed the EGF-R, Caco-2 cells treated or not with EGF were lysed. Each lysate was incubated with either control antibodies or anti-EGF-R antibodies. The immunoprecipitated proteins were submitted to SDS-PAGE and blotted with either anti-P-tyrosine or anti-EGF-R antibodies. EGF treatment, did not modify the amount of EGF-R (right panel of Fig. 2B) whereas it significantly increased the interaction of EGF-R with anti-P-tyrosine antibodies (left panel of Fig. 2B). This experiment proved that EGF strongly increased tyrosine phosphorylation of EGF-R without affecting the level of this protein.

3.4. Tyrosine phosphorylation of c-erb B2

In the experiments to study total protein tyrosine phosphorylation (Figs. 1 and 2A) EGF induced tyrosine phosphorylation of a 185.000 mol.wt. protein we have tentatively identified with c-erb B2. To confirm this identification lysates from Caco-2 cells treated or not with EGF were incubated either with control antibodies or anti-c-erb B2 antibodies. The immunoprecipitated proteins were submitted to SDS-PAGE and blotted with either anti-P-tyrosine or anti-c-erb B2 antibodies. As in the case of EGF-R, EGF treatment did not modify the amount of c-erb B2 whereas significantly stimulated tyrosine phosphorylation of this protein (Fig. 3).

3.5. Analysis of ras-GAP-associated proteins

Untreated or EGF-treated Caco-2 cells were lysed and lysates submitted to immunoprecipitation by either control or anti-ras-GAP antibodies. The immunoprecipitates were analyzed by SDS-PAGE and finally blotted with anti-ras-GAP antibodies. The left panel of Fig. 4A shows that the 120.000 mol.wt. ras-GAP was specifically immunoprecipitated by anti-

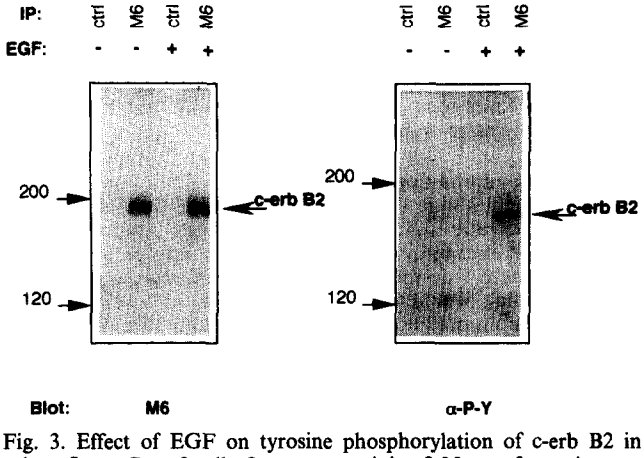

Fig. 3. Effect of EGF on tyrosine phosphorylation of c-erb B2 in subconfluent Caco-2 cells. Lysates containing 2.25 mg of protein were immunoprecipitated by either control (Ctrl) or anti-c-erb B2 (M6) anti-bodies and proteins blotted with either anti-P-tyrosine (α-P-Y) or anti-c-erb B2 antibodies (M6). Mol.wt. markers (×10^{-3}) are indicated at the left. The position of c-erb B2 is indicated at the right.

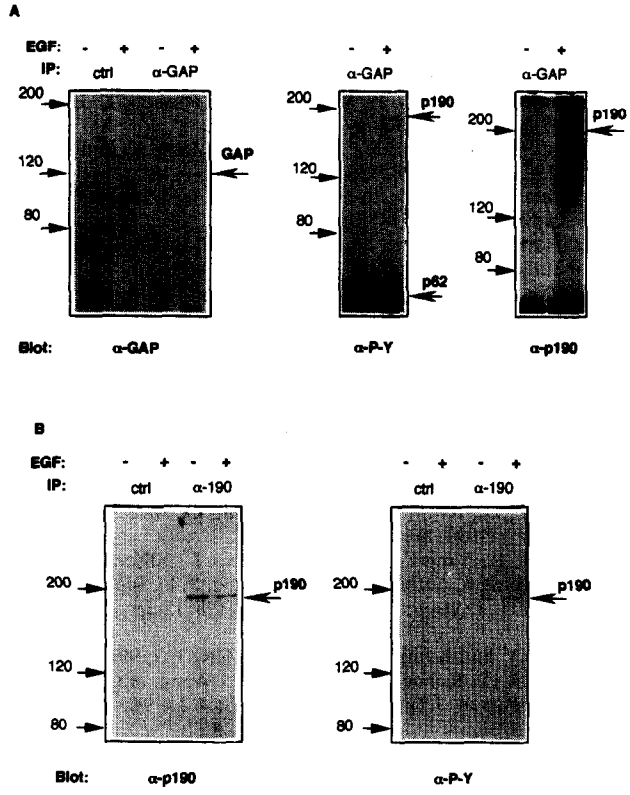

Fig. 4. Effect of EGF on protein association to ras-GAP in subconfluent Caco-2 cells. Lysates (4.5 mg) from serum-starved cells, untreated or treated for 5 min with 200 ng·ml^{-1} of EGF were used. (A) proteins were immunoprecipitated with anti-ras-GAP antibodies and blotted with either anti-ras-GAP (α-GAP) or anti-P-tyrosine (α-P-Y) or anti-p190 antibodies (α-p190). Immunoprecipitation with control antibodies (ctrl) was run in parallel and the precipitate was used for blot with anti-GAP antibodies. (B) proteins were submitted to immunoprecipitation with either control or anti-p190 antibodies. The precipitates were blotted with either anti-p190 (α-p190) or anti-P-tyrosine (α-P-Y) antibodies. Mol.wt. markers (×10^{-3}) are indicated at the left, the position of GAP, p190 and putative p62 are indicated at the right.

ras-GAP antibody and EGF treatment of cells did not affect the level of this protein. When a different aliquot of the same lysate sample immunoprecipitated by anti-ras-GAP antibodies was blotted with anti-P-tyrosine antibody, it became evident that EGF induced the appearance of two ras-GAP-associated tyrosine posphorylated proteins migrating at 62.000 and 190.000 mol.wt. (middle panel of Fig. 4A). This result posed the question: did EGF induce tyrosine phosphorylation of two proteins already associated with ras-GAP before EGF treatment or did it induce association of two tyrosine phosphorylated proteins to ras-GAP, or did EGF induce both tyrosine phosphorylation and ras-GAP association of the two proteins? To answer these questions lysates from EGF-treated and untreated cells were immunoprecipitated with anti-ras-GAP antibodies, submitted to SDS-PAGE and blotted with anti-p190 antibodies. The right panel in Fig. 4A shows that only in cells treated with EGF was p190 associated with ras-GAP. Furthermore cell lysates were immunoprecipitated with control or anti-p190 antibodies, proteins were electrophoretically separated and finally blotted with either anti-p190 or anti-P-tyrosine antibodies. The right panel in Fig. 4B shows that p190 was tyrosine-phosphorylated under basal conditions. Since the level of p190 was slightly lower in the sample of EGF-treated cells (left panel in Fig. 4B) whereas tyrosine phosphorylation was stronger in the same sample (right panel in Fig. 4B), taken together the findings indicated that EGF increased basal tyrosine phosphorylation of the p190 protein.

4. Discussion

EGF is a factor that regulates growth, cell migration, cell–cell interaction, expression of digestive enzymes of intestinal cells [8,18]. This multiplicity of effects poses the question of how EGF can modulate these different processes. The present study demonstrates that EGF is able to strongly stimulate EGF-R phosphorylation of subconfluent proliferating Caco-2 cells. It has recently been reported that EGF stimulates the appearance of tyrosine phosphorylated EGF-R in suckling rat jejunum [19]. Stimulation of EGF-R tyrosine phosphorylation is a crucial process for the EGF action since it determines the affinity and/or the specificity of the receptor-intrinsic kinase for its cellular substrates [20] and tyrosine phosphorylation of the substrates is thought to represent the initial step of intracellular mitogenetic signal transduction. An EGF dose–response analysis has determined the concentrations at which EGF induces optimal tyrosine phosphorylation of different proteins in Caco-2 cells. Among the putative substrates of EGF-R we observed a tyrosine phosphorylated protein with mol.wt of 185.000 we identified with c-erb B2. This protein is related to the EGF-R [21] and its tyrosine phosphorylation is stimulated by EGF [22]. c-erb B2 is likely to be a physiological substrate of EGF-R. The interaction between EGF-R and c-erb B2 is unique in representing a circuit in which one receptor appears to be directly phosphorylated by another in vivo and is the first example of a tyrosine kinase cascade in which both enzymes have been identified [22].

The EGF-dependence of p190 association with ras-GAP we observe in Caco-2 cells has been previously studied with conflicting results in other systems. In fact, while some authors observe equal amounts of p190 and p62 proteins in ras-GAP complexes before and after cell EGF treatment [12], others have observed complexes of ras-GAP with p62 and p190 proteins after EGF treatment alone [11,23]. The ras-GAP–p190 complex reduces the GTP-ase activating activity of ras-GAP therefore increasing the active form of ras (ras-GTP) [12]. In addition, p190 contains different domains; one of them is located in the C-terminus [10] and acts as a GAP specific for rho family proteins [13]. Therefore, the complex we observe between ras-GAP and p190 may allow coupling of EGF stimulated signals to both ras and rho GTP-ases.

EGF stimulates tyrosine phosphorylation of p190 in subconfluent Caco-2 cells. This stimulation could be responsible for the association of p190 with the SH2 domains of ras-GAP [11]. In EGF-treated cells we observe association of a tyrosine phosphorylated protein with mol.wt. of 62.000 mol.wt. to ras-GAP. This is most likely to be the p62 protein which has been found in complex with ras-GAP together with p190 [11,12]. It has been suggested that this plays a role in DNA and RNA metabolism [17].

Taken together our findings are consistent with the interpretation that in subconfluent Caco-2 cells EGF activation of the EGF-R tyrosine kinase and phosphorylation of critical substrates are required for the formation of ras-GAP–p190 com-

plex. This complex could have a crucial role for the EGF action on growth, cell-cell interaction motility and polarity of proliferating intestinal cells.

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