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Receptor for Bombesin with Associated Tyrosine Kinase Activity

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The neuropeptide bombesin is known for its potent mitogenic activity on murine 3T3 fibroblasts and other cells. Recently it has been implicated in the pathogenesis of small cell lung carcinoma, in which it acts through an autocrine loop of growth stimulation. Phosphotyrosine (P-Tyr) antibodies have been successfully used to recognize the autophosphorylated receptors for known growth factors. In Swiss 3T3 fibroblasts, phosphotyrosine antibodies identified a 115,000-M_r cell surface protein (p115) that became phosphorylated on tyrosine as a specific response to bombesin stimulation of quiescent cells. The extent of phosphorylation was dose dependent and correlated with the mitogenic effect induced by bombesin, measured by [^{3}H]thymidine incorporation. Tyrosine phosphorylation of p115 was detectable minutes after the addition of bombesin, and its time course paralleled that described for the binding of bombesin to its receptor. Immunocomplexes of phosphorylated p115 and phosphotyrosine antibodies bound ^{125}I-labeled [Tyr^4]bombesin in a specific and saturable manner and displayed an associated tyrosine kinase activity enhanced by bombesin. Furthermore, the ^{125}I-labeled bombesin analog gastrin-releasing peptide, bound to intact live cells, was coprecipitated with p115. These data strongly suggest that p115 participates in the structure and function of the surface receptor for bombesin, a new member of the family of growth factor receptors with associated tyrosine kinase activity.

Many recent reports on proliferative growth factors provide evidence that peptides already known for their hormonal or neurotransmitter nature can act as mitogens. Two mammalian members of the tachykinin family, substance P and substance K, which are present in sensory neurons, specifically stimulate DNA synthesis in cultured arterial smooth muscle cells and human skin fibroblasts (32). The neurohormone vasopressin and the tetradecapeptide bombesin (1) are also potent mitogens. The former stimulates DNA synthesis in chondrocytes (26), bone marrow cells (18), and Swiss 3T3 mouse fibroblasts (34). The latter, which stimulates hormone secretion (37) and smooth muscle contraction and affects temperature control and behavior in mammals (14, 38), shows potent mitogenic activity on Swiss 3T3 fibroblasts (35), in which high-affinity receptors for peptides of the bombesin family were recently demonstrated (45).

Bombesin and bombesinlike peptides have been detected in various normal mammalian tissues (28, 40, 42), together with specific cell surface receptors (21, 27). These peptides have also been shown to behave as growth factors for normal human bronchial epithelial cells (44) and for human small cell lung carcinoma, in which they appear to act in an autocrine circuit stimulating growth (6). Furthermore, high levels of bombesinlike immunoreactivity were reported in rat mammary (13) human lung (10, 29), and human thyroid (25) tumors. The broad tissue specificity of this peptide and its mitogenic activity on different cell types suggest a role for bombesin in growth control of both normal and transformed cells.

Known growth factors trigger one or more of the following events in target cells: (i) activation of receptor-associated tyrosine protein kinase activity (20), (ii) stimulation of polyphosphoinositide breakdown, leading to protein kinase C activation and Ca^{2+} mobilization (2, 24), (iii) alkalinization of cytoplasm through the activation of an Na^+-K^+ antiport (15, 23, 30), and (iv) expression of c-*fos* and c-*myc* proto-oncogenes (16, 17). Bombesin, like the tachykinins and vasopressin, stimulates polyphosphoinositide breakdown (2). It has also recently been shown that bombesin induces rapid and transient expression of c-*fos* and c-*myc* proto-oncogenes (A. P. Palumbo, P. Rossino, and P. M. Comoglio, Exp. Cell Res., in press).

To assess whether the mitogenic effect of bombesin involves a receptor-linked protein tyrosine kinase activity, we used purified antibodies capable of recognizing phosphotyrosine (P-Tyr) residues in proteins (5, 31, 46) (P-Tyr antibodies). These antibodies have been used to detect tyrosine protein kinases associated with growth factor receptors through identification of the autophosphorylated receptor and its cellular substrates in mitogen-stimulated cells (7a, 9, 11, 12, 43). The present investigation demonstrates that bombesin specifically stimulates the phosphorylation on tyrosine of a membrane protein of M_r 115,000 (p115). Immunoprecipitates of this protein, made with P-Tyr antibodies from bombesin-stimulated cells, displayed a bombesin-dependent tyrosine kinase activity and bound labeled bombesin in a specific and saturable manner. When the bombesin analog gastrin-releasing peptide (GRP) was bound to live intact cells, it coprecipitated with p115.

MATERIALS AND METHODS

Cells and reagents. Swiss 3T3 mouse fibroblasts were obtained from L. Alberghina (Milan) and grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Flow Laboratories) in a 5% CO_2, water-saturated atmosphere. Growth-arrested, confluent monolayers were prepared as follows. Cells were seeded at a density of 3,000 cells per cm^2, fresh medium was added after 3 days, and the monolayers were used after at least 4 days. The BALB/c 3T3 cell line was obtained from the American Type Culture Collection and grown as above. Growth-arrested monolayers were prepared by 24 h of serum starvation (0.2%).

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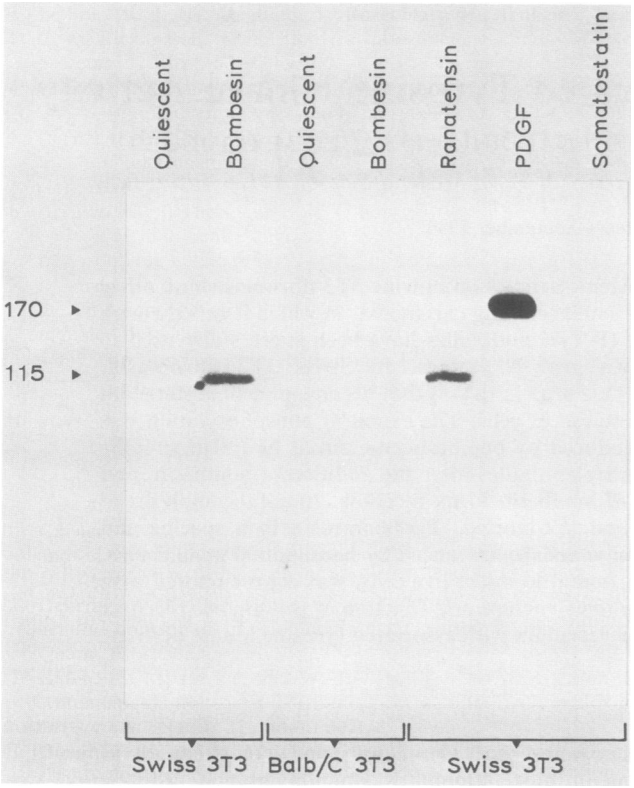

FIG. 1. Proteins labeled by P-Tyr antibodies in Western blots of quiescent Swiss 3T3 or BALB/c 3T3 fibroblasts either unstimulated or exposed to different peptides at 37°C. Bombesin, ranatensin, and somatostatin were added at 3 nM for 30 min; partially purified PDGF was added at 40 μg/ml for 10 min. Intact cells were directly solubilized in boiling Laemmli buffer. Samples were adjusted to an equal protein content of approximately 200 μg per lane and subjected to SDS-PAGE and Western blot analysis with P-Tyr antibodies and ^{125}I-labeled *S. aureus* protein A. The approximate M_r (in thousands) of the labeled proteins is shown at the left. Autoradiogram from a 24-h exposure with an intensifying screen.

Bombesin, ranatensin, and somatostatin were obtained from Sigma Chemical Co. Partially purified platelet-derived growth factor (PDGF) was a gift of E. Sturani (Milan).

Antibodies. P-Tyr antibodies were raised in rabbits immunized with azobenzylphosphonate (a phosphatase-resistant synthetic analog of P-Tyr) covalently coupled to keyhole limpet hemocyanin (33). Antibodies were affinity purified on P-Tyr-BSA (bovine serum albumin derivatized with P-Tyr by incubation with *N*-ethyl-*N'*-3-dimethylaminopropylcarbodiimide) (9) coupled to cyanogen bromide-activated Sepharose 4B.

Western blotting. Growth-arrested fibroblasts, quiescent or stimulated, were washed in phosphate-buffered saline (pH 7.4) and solubilized in boiling Laemmli buffer (22). Samples were adjusted to an equal protein content of approximately 200 μg, electrophoresed onto a 5 to 15% polyacrylamide gradient slab gel, and transferred to nitrocellulose sheets as previously described (3, 39). Blots were probed with 12 μg of purified antibodies per ml, followed by ^{125}I-labeled *Staphylococcus aureus* protein A (Amersham Corp.). Autoradiograms were exposed with an intensifying screen for 1 to 2 days. The M_r or labeled proteins was estimated relative to the electrophoretic mobility of cotransferred *methyl*-^{14}C-labeled protein standards (Amersham).

Extracellular proteolysis. Swiss 3T3 monolayers were exposed to 3 nM bombesin for 30 min, washed several times with serum-free DMEM, and then incubated with various concentrations of trypsin or pronase in serum-free DMEM at 37°C. Proteolytic activity was stopped at the indicated time by the addition of specific inhibitors or excess serum, and the cells were rapidly collected and solubilized in boiling Laemmli buffer for Western blotting analysis. A dye exclusion test performed on cells treated in the same way confirmed the preservation of the membrane permeability barrier. Controls included stimulated cells washed and incubated for 20 min without proteases.

Cell extraction and phosphorylation of detergent-insoluble cell matrices. Quiescent monolayers were either stimulated with bombesin 3 nM for various times or used directly. They were washed twice in an ice-cold buffer (pH 7.2) containing 10 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)], 100 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose, and 5 mM EGTA (buffer A). The cells were then extracted with the same buffer plus 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 kallikrein-inhibiting unit (KIU) of aprotinin per ml, 50 μg of leupeptin per ml, and 4 μg of pepstatin per ml to inhibit proteases, and 0.1 mM Na_3VO_4, 1 mM $ZnCl_2$, and 5 mM *O*-phospho-L-tyrosine (buffer B) to inhibit P-Tyr phosphatases for 5 min at 0°C. Detergent-insoluble cell fractions were labeled in a minimal volume of extraction buffer supplemented with 10 mM $MnCl_2$, 3 nM bombesin, and 500 μCi of [γ-^{32}P]ATP (specific activity, 7,000 Ci/mmol; New England Nuclear Corp.) per ml for 5 min at 0°C. After removal of the reaction buffer, labeled cell fractions were dissolved in excess RIPA buffer (50 mM Tris hydrochloride, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 5 mM EDTA and 1 mM PMSF, 100 KIU of aprotinin per ml, 50 μg of leupeptin per ml, 4 μg of pepstatin per ml, 0.1 mM Na_3VO_4, 0.1 mM $ZnCl_2$, and 0.1% BSA. After a brief sonication, the extract was cleared at 10,000 × *g* for 20 min and immunoprecipitated with 16 μg of either purified P-Tyr antibodies or normal rabbit immuno-

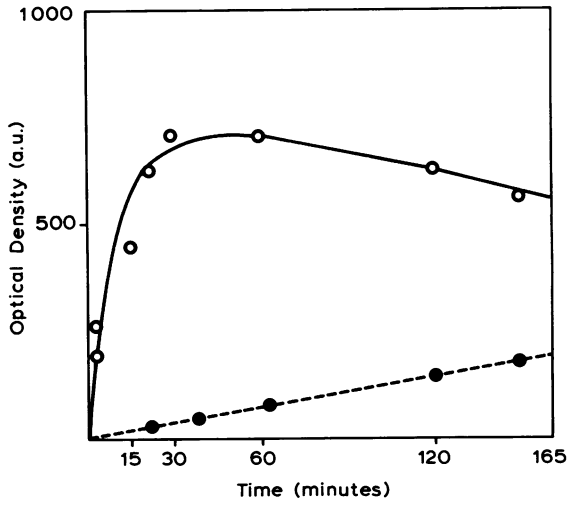

FIG. 2. Time course of p115 tyrosine phosphorylation in cells exposed to 3 nM bombesin at 37°C (solid line) and at 4°C (dotted line). The intensity of the p115 band visualized by autoradiography after Western blot analysis with P-Tyr antibodies and ^{125}I-labeled protein A was quantitated with a densitometer. Values shown are from a representative experiment. a.u., Absorbance units.

globulins per ml for 2 h at 4°C as described previously (5). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography with an intensifying screen for 6 h. Molecular weights were estimated relative to the electrophoretic mobility of *methyl*-^{14}C-labeled protein standards (Amersham). The single ^{32}P-labeled bands were quantitated with a beta-scanner apparatus (Ambis).

Phosphoamino acid analysis. For phosphoamino acid analysis, ^{32}P-labeled p115 was eluted from the gel in a solution containing 50 mM NH_4HCO_3, 0.1% SDS, 2% 2-mercaptoethanol, and 10 μg of BSA per ml. The eluate was lyophilized, redissolved in water, and precipitated with trichloroacetic acid. The precipitate was washed with ethanol and air dried. Acid hydrolysis was performed for 1 h in 6 N HCl (constant boiling; Pierce Chemical Co.) at 115°C under vacuum. The hydrolysate was washed several times with water, redissolved in TLE buffer (pyridine-acetic acid-water, 5:50:945, pH 3.5) and spotted together with phosphoamino acid standards onto thin-layer silica gel plates for electrophoresis at 1,100 V. Plates were sprayed with ninhydrin to visualize phosphoamino acid standards and exposed for autoradiography with an intensifying screen for 7 days.

Metabolic labeling with [^{35}S]methionine and immunoprecipitation. Quiescent 3T3 cells were labeled for 18 h with 50 μCi of [^{35}S]methionine (Amersham) per ml in spent medium dialyzed against methionine-free DMEM. Monolayers were then stimulated with bombesin (3 nM) for 30 min, washed twice in buffer A, and extracted with buffer B lacking P-Tyr and with 3 nM of bombesin for 5 min at 0°C. The cell extract was dialyzed against buffer B to remove the nucleotide pool, which antagonizes the immunoprecipitation of proteins by P-Tyr antibodies (5, 11), cleared, and immunoprecipitated as described above. A control immunoprecipitation was performed from [^{35}S]methionine-labeled unstimulated cells. Immunocomplexes were collected on protein A-Sepharose, washed four times in buffer B, and eluted in Laemmli buffer for SDS-PAGE. After fluorography, the gel was exposed for autoradiography for 2 days. Molecular weights were estimated relative to the electrophoretic mobility of *methyl*-^{14}C-labeled protein standards (Amersham).

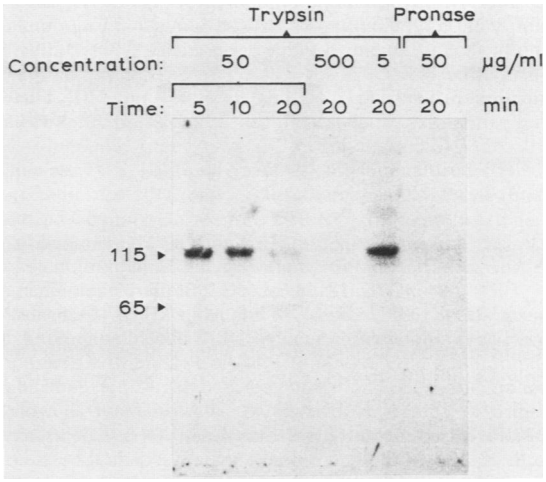

FIG. 4. Susceptibility of p115 to extracellular proteolysis. Confluent Swiss 3T3 monolayers were stimulated with 3 nM bombesin for 30 min, washed, and exposed to different concentrations of trypsin or of the protease mixture pronase at 37°C for the time indicated at the top of each lane. Proteolysis was stopped by the addition of an equimolar amount of soybean trypsin inhibitor or of excess serum, and the cells were directly solubilized in boiling Laemmli buffer for Western blot analysis with P-Tyr antibodies. The approximately M_r (in thousands) of the labeled proteins is shown. Autoradiogram from a 36-h exposure with an intensifying screen.

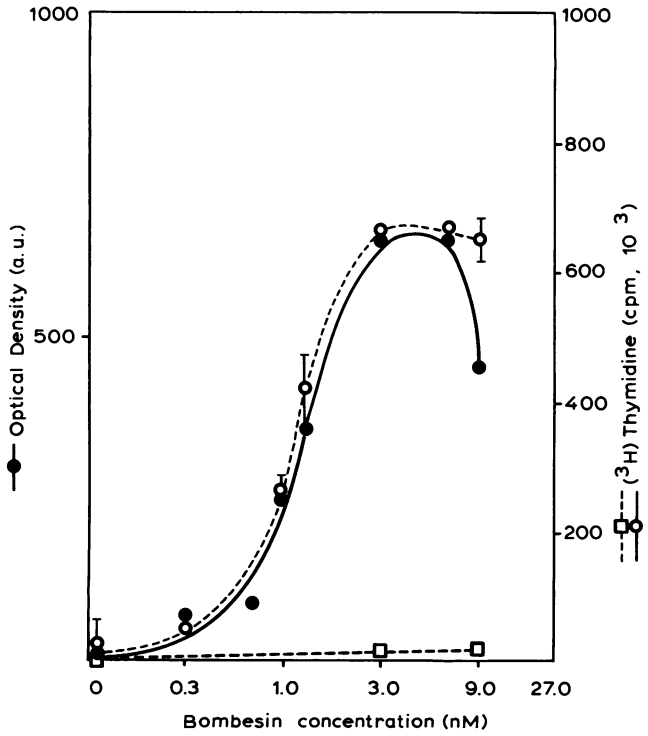

FIG. 3. Dose dependence of bombesin-induced p115 tyrosine phosphorylation (●——●) and [^{3}H]thymidine incorporation in Swiss 3T3 cells (○---○). The extent of response was assayed after 30 min at 37°C for phosphorylation and after 42 h in serum-free medium for [^{3}H]thymidine incorporation. The intensity of the p115 band visualized by autoradiography after Western blot analysis with P-Tyr antibodies and ^{125}I-labeled protein A was quantitated with a densitometer. Values shown are from representative experiments. No mitogenic effect was exerted by bombesin on control BALB/c 3T3 cells (□---□).

Immunocomplex kinase assay. Quiescent 3T3 cells were stimulated with 3 nM bombesin for 30 min, washed, extracted with buffer B, and immunoprecipitated as described above. Immunocomplexes were collected on protein A-Sepharose, washed four times in buffer B without bombesin, and phosphorylated in 40 μl of the same buffer supplemented with 10 mM $MnCl_2$ and 15 μCi of [γ-^{32}P]ATP at 30°C for 5 min in the presence or absence of 3 nM bombesin. The reaction was stopped by dilution in excess ice-cold buffer B containing 10 mM EDTA, and the immunocomplexes were further washed twice in the same buffer. Samples were eluted from the protein A-Sepharose by boiling in Laemmli buffer and subjected to SDS-PAGE, autoradiography with an intensifying screen for 2 h, and phosphoamino acid analysis as described above.

Binding to immunocomplexes. For binding studies, increasing amounts of ^{125}I-labeled [Tyr^4]bombesin (1.5×10^6 cpm/pmol) were added to immunoprecipitates prepared as described above with either P-Tyr antibodies or normal rabbit immunoglobulins from bombesin-stimulated cell monolayers and incubated overnight at 4°C in 50 μl of buffer B. The immunoprecipitates were then washed twice with

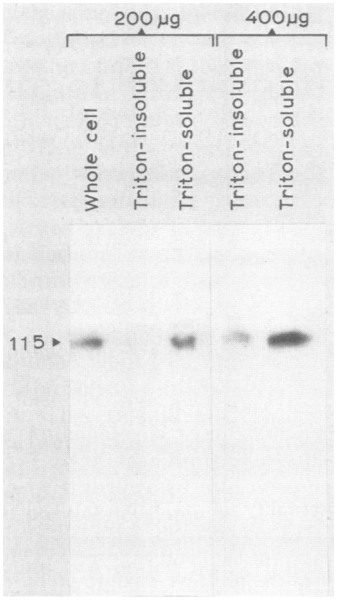

FIG. 5. Fractionation of tyrosine-phosphorylated p115 by its solubility in a buffer containing 1% Triton X-100 and 1 mM $ZnCl_2$. Confluent Swiss 3T3 monolayers were stimulated with 3 nM bombesin for 30 min and either directly solubilized in Laemmli buffer (whole cells) or washed with buffer A and extracted with buffer B as detailed in Materials and Methods. The Triton-soluble cell extract was lyophilized and dissolved in Laemmli buffer. The Triton-insoluble cell matrices were directly solubilized in Laemmli buffer. All samples were analyzed for protein concentration by a modification of the Lowry method (16a), and the amount indicated at the top of each lane was subjected to SDS-PAGE and Western blot analysis with P-Tyr antibodies. The approximate M_r (in thousands) of the labeled proteins is shown. Autoradiogram from a 30-h exposure with an intensifying screen.

excess buffer B and counted for bound radioactivity in a Packard gamma counter. Specific binding was calculated by subtracting counts bound in the presence of excess (1 μM) unlabeled bombesin.

Binding to cells. Quiescent Swiss 3T3 monolayers were washed twice in DMEM-RPMI media (1:1) with BSA (1 mg/ml) and incubated in the same medium with 2 nM ^{125}I-GRP (specific activity, 2,000 Ci/mmol; Amersham) for 30 min at 37°C in a 5% CO_2, water-saturated atmosphere. Cells were washed twice in buffer A, extracted in buffer B for 5 min at 0°C, and immunoprecipitated in the absence of bombesin with P-Tyr antibodies or control pooled rabbit immunoglobulins as described above. The immunoprecipitates were washed twice in buffer A and incubated with or without 1 μM unlabeled bombesin for 1 h at 4°C. After further washings the immunoprecipitates were counted in a Packard gamma counter. Specific binding was calculated by subtracting counts bound in the presence of excess bombesin.

RESULTS

Phosphorylation on tyrosine of an M_r-115,000 protein in Swiss 3T3 cells. Swiss 3T3 monolayers were grown to confluence and used at least 3 days after the last change of medium: under these conditions the cells cease proliferation and enter a quiescent phase. Addition of 3 nM bombesin promoted DNA synthesis in a significant fraction of the cells in the absence of any other growth factor, as assessed by [^{3}H]thymidine incorporation (see Fig. 3) (35). To assay the induction of tyrosine phosphorylation in cellular proteins by bombesin stimulation of intact cells, growth-arrested monolayers were incubated with a mitogenic concentration of bombesin or an equimolar amount of different control peptides in the absence of any other growth factor. At different times after addition, monolayers were processed for Western blot analysis with purified P-Tyr antibodies and ^{125}I-labeled protein A. Control experiments with preimmune rabbit immunoglobulins consistently showed no binding (data not shown). While no protein was labeled by P-Tyr antibodies in quiescent unstimulated Swiss 3T3 cells, a protein with an apparent M_r of 115,000 (p115) was strongly labeled in bombesin-stimulated cells (Fig. 1).

The specificity of this effect was assayed by exposing different cell types to bombesin and Swiss 3T3 fibroblasts to different peptides. When bombesin was added to serum-starved BALB/c 3T3 fibroblasts, a cell line not bearing bombesin receptors (45), no protein labeling by P-Tyr antibodies was induced. On the other hand, p115 was clearly labeled in Swiss 3T3 cells stimulated with a mitogenic concentration of the bombesin analog ranatensin (Fig. 1) or GRP (not shown), while no protein was labeled when the same cells were exposed to the unrelated nonmitogenic peptide somatostatin (Fig. 1). When another growth factor effective on Swiss 3T3 fibroblasts was added to these cells, a protein with the M_r expected for its receptor (8, 9, 12) was labeled by P-Tyr antibodies. The PDGF-induced autophosphorylation of the 170-kilodalton PDGF receptor is shown in Fig. 1.

Time and dose dependence of bombesin-induced p115 tyrosine phosphorylation in intact cells. To study the time course

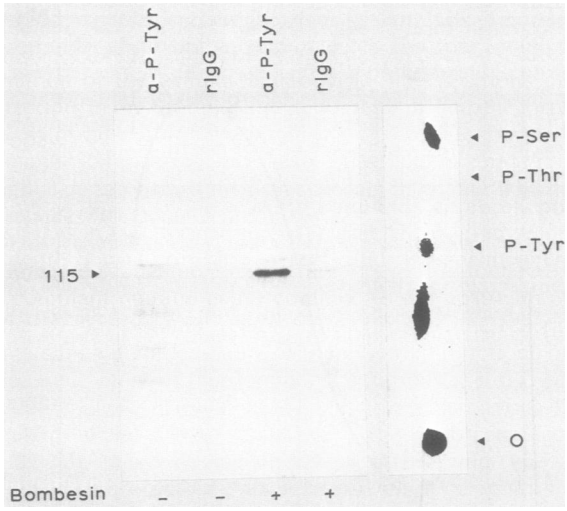

FIG. 6. Bombesin-dependent phosphorylation of p115 in detergent-permeabilized cells. Quiescent 3T3 monolayers were extracted with Triton X-100 in buffer B and incubated with [γ-^{32}P]ATP in the presence (+) or absence (−) of bombesin. Labeled proteins were solubilized in RIPA buffer, immunoprecipitated with either P-Tyr antibodies (a-P-Tyr) or pooled normal rabbit immunoglobulins (rIgG), and analyzed by SDS-PAGE and autoradiography (6 h with intensifying screen). The approximate M_r (in thousands) of the labeled proteins is shown. One-dimensional phosphoamino acid analysis of the immunoprecipitated ^{32}P-labeled p115 is shown. The origin (O) and the electrophoretic mobility of phosphoamino acid standards are indicated. Autoradiogram from a 1-week exposure with intensifying screen. P-Ser and P-Thr, phosphoserine and phosphothreonine, respectively.

of p115 tyrosine phosphorylation, growth-arrested Swiss 3T3 monolayers were exposed to bombesin for different times and analyzed by Western blot with P-Tyr antibodies. The intensity of the p115 band visualized by autoradiography was quantitated by densitometry. When bombesin treatment was performed at 37°C, p115 phosphorylation was already detectable after 2 min of exposure, the earliest time assayed, and its extent increased thereafter. A plateau was reached after 30 min of stimulation and maintained for approximately 1 h, after which p115 labeling decreased (Fig. 2). When intact cells were exposed to bombesin at 4°C, p115 phosphorylation displayed much slower kinetics.

The dependence of p115 tyrosine phosphorylation on bombesin concentration was similarly investigated. Incubation time was 30 min at 37°C for every concentration tested, as suggested by the kinetics described above. p115 tyrosine phosphorylation could be detected by P-Tyr antibodies at a bombesin concentration of 1 nM and was maximal at 3 nM. A plateau was observed between 3 and 6 nM, while the extent of p115 tyrosine phosphorylation decreased with higher bombesin concentrations. This could be ascribed to desensitization or to internalization of ligand-receptor complexes as reported for other receptors (20) (Fig. 3, solid line). This dose dependence was superimposible with that observed for the mitogenic effect of bombesin on Swiss 3T3 cells, as assessed by [^{3}H]thymidine incorporation (Fig. 3, dotted line).

Susceptibility of p115 to extracellular proteolysis. To investigate the plasma membrane association of p115, we tested its susceptibility in intact cells to proteases added to the extracellular fluid. The tyrosine-phosphorylated p115 disappeared in Western blots of cells treated with trypsin (50 μg/ml) for 20 min at 37°C. This also happened with the more broadly acting enzyme mixture pronase at 50 μg/ml. In the mildest but still effective conditions of trypsin cleavage, a faint immunolabeling of a lower-molecular-weight species, of approximately M_r 65,000, accompanied the disappearance of the p115 band (Fig. 4). It should be remembered that the antibodies detect p115 only via its phosphorylated tyrosine residue(s). Rapid dephosphorylation of the intracellular fragment probably occurs shortly after proteolytic cleavage. Under the conditions used, protease treatment of intact cells affected only proteins exposed to the outer cell surface, as assessed by cell viability tests. Moreover, labeling of Western blots with antibodies against known intracellular proteins (e.g., vinculin, p36) did not show any detectable proteolysis (data not shown).

Bombesin-dependent tyrosine phosphorylation of p115 in detergent-permeabilized cells. Cellular proteins from growth-arrested monolayers stimulated with bombesin were fractionated by their solubility in a buffer containing 1% nonionic detergent, 1 mM Zn^{2+}, and protease and phosphatase inhibitors. Due to the presence of the membrane-stabilizing Zn^{2+} (41) in the extraction buffer, the detergent-insoluble fraction contained approximately 40% of total cellular proteins. Western blotting analysis showed that the tyrosine-phosphorylated p115 was preferentially detergent extracted. However, a smaller amount could be demonstrated in the detergent-insoluble matrices by overloading the sample (Fig. 5).

This enabled us to design an in situ phosphorylation assay, in which [γ-^{32}P]ATP and Mn^{2+} ions were added either with or without bombesin to detergent-insoluble cell matrices as described in detail elsewhere (4, 5). The labeled cell matrices were then washed free of the reaction buffer and solubilized in RIPA buffer for immunoprecipitation with P-Tyr antibodies. ^{32}P-labeled p115 was precipitated by P-Tyr antibodies

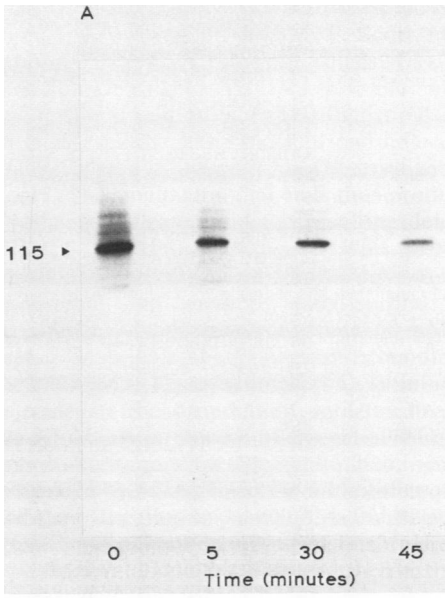

FIG. 7. Dependence of p115 in situ phosphorylation on previous in vivo exposure of the cells to bombesin. Quiescent 3T3 cells were either stimulated with 3 nM bombesin for the indicated time or used directly (time zero). Monolayers were extracted with buffer B, phosphorylated in the presence of bombesin, and immunoprecipitated with P-Tyr antibodies. (A) Autoradiogram from a 6-h exposure with intensifying screen. The approximate M_r (in thousands) of the labeled proteins is shown. (B) Amount of ^{32}P incorporated into the p115 band was quantitated directly in the gel with a beta-scanner apparatus.

only from cell matrices exposed to [γ-^{32}P]ATP in the presence of 3 nM bombesin. After identification by autoradiography, the immunoprecipitated ^{32}P-labeled p115 was excised from the gel and eluted for phosphoamino acid analysis, which confirmed the presence of P-Tyr, although a substantial amount of phosphoserine was also noted (Fig. 6).

The extent of bombesin-dependent p115 phosphorylation in the in situ assay was also affected by previous exposure of the cell monolayers to bombesin. The longer the exposure,

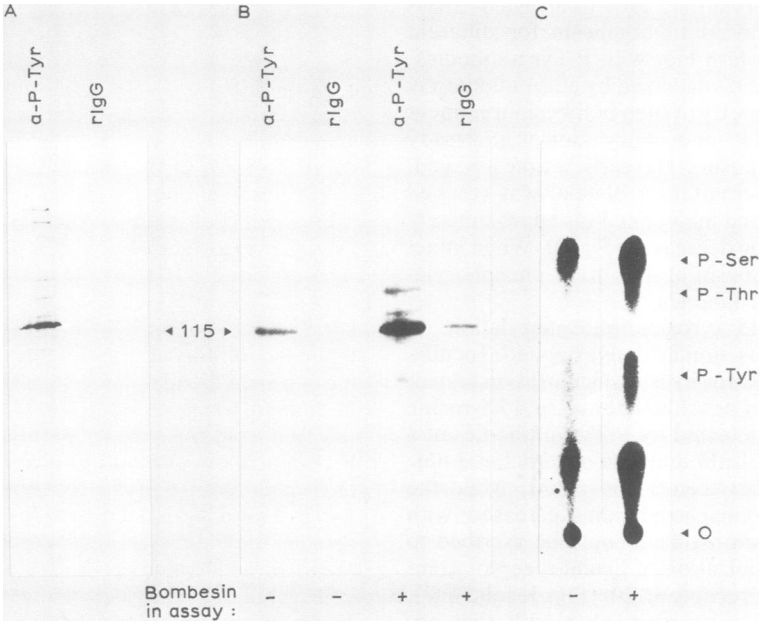

FIG. 8. Immunoprecipitates of p115 display tyrosine kinase activity dependent on bombesin and phosphorylating p115. Quiescent 3T3 monolayers were stimulated with bombesin and extracted with Triton X-100 in buffer B containing bombesin. The extract was cleared, dialyzed against buffer B, and immunoprecipitated by P-Tyr antibodies (a-P-Tyr) or pooled normal rabbit immunoglobulins (rIgG). (A) Immunoprecipitates were prepared from cells metabolically labeled with [^{35}S]methionine and analyzed by SDS-PAGE, fluorography, and autoradiography (48-h exposure). (B) Kinase activity associated with the immunoprecipitates was assayed with [γ-^{32}P]ATP in the absence (−) and presence (+) of bombesin in the reaction buffer. Labeled proteins were analyzed by SDS-PAGE and autoradiography (2-h exposure with an intensifying screen). The approximate M_r (in thousands) of the labeled proteins is shown. (C) One-dimensional phosphoamino acid analysis of ^{32}P-labeled p115 phosphorylated in the absence (−) and presence (+) of bombesin. The origin (O) and the electrophoretic mobility of phosphoamino acid standards are indicated. Autoradiogram from a 1-week exposure with intensifying screen. P-Ser and P-Thr, Phosphoserine and phosphothreonine, respectively.

the lower was the extent of p115 phosphorylation. Maximal ^{32}P labeling of p115 was obtained when the cells were not exposed to bombesin before the phosphorylation assay (Fig. 7). The decrease in the extent of p115 phosphorylation with increasing time of exposure to the agonist in vivo may suggest the occurrence of bombesin-induced desensitization or downregulation of p115. Since bombesin stimulates the phosphorylation of p115 on tyrosine, an alternate explanation is that an increasing number of the available phosphorylation sites become occupied before the cells are extracted. However, it is more likely that the sites of phosphorylation available in vitro differ from those available in intact cells, as discussed below.

Bombesin-dependent tyrosine kinase activity. Growth-arrested Swiss 3T3 monolayers were stimulated with 3 nM bombesin and extracted with 1% nonionic detergent in a buffer containing phosphatase and protease inhibitors and supplemented with 3 nM bombesin. The extract was cleared, dialyzed to remove the cellular nucleotide pool (which antagonizes the precipitation of P-Tyr-containing proteins by P-Tyr antibodies) (5, 11), and immunoprecipitated with either P-Tyr antibodies or control rabbit immunoglobulins. P-Tyr antibodies specifically precipitated p115 as a major molecular species from cells metabolically labeled with [^{35}S]methionine and stimulated with bombesin (Fig. 8A). No protein was specifically precipitated from unstimulated cells (not shown).

When the immunoprecipitates were exposed to [γ-^{32}P]ATP and Mn^{2+} to assay for kinase activity, p115 became phosphorylated. The responsible enzymatic activity was highly enhanced by addition of bombesin to the reaction buffer (Fig. 8B). Phosphoamino acid analysis showed phosphate incorporation into both tyrosine and serine residues. However, the P-Tyr content was much higher in the p115 phosphorylated in the presence of bombesin. On the other hand, the amount of phosphoserine seemed not to be affected by bombesin, indicating that the bombesin-dependent phosphorylation of p115 occurs mainly on tyrosine residues (Fig. 8C). In analogy with the other known tyrosine kinases (17, 19) and in view of the high p115 content of the immunoprecipitate, one can speculate on a bombesin-dependent tyrosine autophosphorylation of p115. There is an apparent paradox in this observation, since p115 immunoprecipitated with P-Tyr antibodies must by definition already be phosphorylated on tyrosine residues prior to the addition of bombesin in the in vitro assay. This, however, can be explained by the occurrence of multiple alternate sites for autophosphorylation, as is the case for the epidermal growth factor (EGF) receptor and other tyrosine kinases (19). Moreover, P-Tyr antibodies were previously shown to allow further in vitro phosphorylation of other precipitated tyrosine kinases (7a, 31). On the other hand, the observed serine phosphorylation of p115 could be ascribed to an unrelated associated kinase activity. Under the extremely permissive reaction conditions used (see Materials and Methods section), it is conceivable that even small amounts of contaminant kinases have such an effect. This has already been shown to occur in immunocomplex kinase assays of other tyrosine kinases (7).

Specific binding to immunoprecipitates of p115 from bom-

besin stimulated cells. ^{125}I-labeled [Tyr^4]bombesin was incubated with immunoprecipitates of p115 prepared as described above from bombesin-stimulated cells. Specific and saturable binding was observed only in the immunoprecipitates made with P-Tyr antibodies. No significant binding of ^{125}I-labeled [Tyr^4]bombesin was observed in control immunocomplexes made with pooled rabbit immunoglobulins (Fig. 9). Specific binding was determined by subtracting from the total counts the radioactivity bound in the presence of excess unlabeled bombesin. Saturation of binding was attained at an approximate concentration of ^{125}I-labeled [Tyr^4]bombesin of 15 nM after overnight incubation at 4°C in the extraction buffer. This result implies the presence of binding sites in the immunocomplex. Presumably these sites become available during the preparation of immunocomplexes by limited dissociation of the unlabeled bombesin used to pretreat the cells or are occupied by exchange with the labeled analog during the following prolonged incubation.

^{125}I-labeled GRP bound to membrane receptors coprecipitated with p115 by P-Tyr antibodies. The bombesin analog GRP, labeled with ^{125}I, was added to confluent 3T3 monolayers to bind to and activate its receptors (45). The cells were extracted with nonionic detergent and immunoprecipitated as above. These conditions appeared to be suitable for preserving a fraction of the ligand-receptor complex (see above) (36). A small but significant amount of radiolabeled GRP was specifically coprecipitated by P-Tyr antibodies in immunocomplexes known to contain largely only p115. The specificity of the binding was assessed by competition experiments performed with an excess of unlabeled bombesin

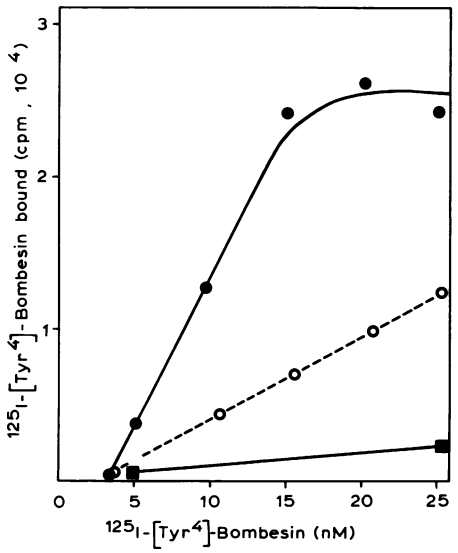

FIG. 9. ^{125}I-labeled [Tyr^4]bombesin binds specifically to immunoprecipitates of p115 from bombesin-stimulated cells. The indicated concentrations of ^{125}I-labeled [Tyr^4]bombesin (specific activity, 1.5 × 10^6 cpm/pmol) were added to immunoprecipitates of p115 prepared as described in the text from bombesin-stimulated cells and incubated overnight at 4°C in buffer A. The immunoprecipitates were then washed with buffer A and counted for bound radioactivity. For immunoprecipitates prepared with P-Tyr antibodies, specific binding (●——●) was calculated by subtracting counts bound in the presence of an excess of unlabeled bombesin (○---○). The observed binding to control immunoprecipitates made with pooled normal rabbit immunoglobulins (■——■) is shown. Values shown are means of duplicate experiments.

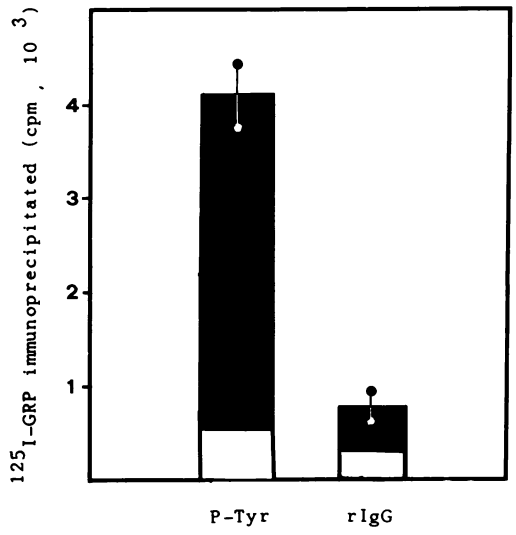

FIG. 10. Bombesin analog GRP bound to membrane receptors of intact cells specifically coprecipitated with p115 by P-Tyr antibodies. Swiss 3T3 monolayers were stimulated with 2 nM ^{125}I-labeled GRP (specific activity, 2 × 10^3 Ci/mmol) for 30 min at 37°C. After repeated washings, the cells were extracted with 1% Triton X-100 in buffer B and immunoprecipitated with either P-Tyr antibodies (P-Tyr) or pooled normal rabbit immunoglobulins (rIgG) as detailed in the Materials and Methods section. The immunoprecipitates were counted for bound radioactivity after a further 1 h of incubation at 0°C in the absence (black and white areas) or presence (white areas only) of a 1,000-fold excess of unlabeled bombesin. Specific binding (black areas) was calculated by subtracting counts bound in the presence of the excess unlabeled bombesin. Values shown are means ± standard error of the mean for triplicate samples.

both on intact cells (not shown) and on the immunoprecipitates. The radiolabeled GRP bound to the P-Tyr antibody immunoprecipitates was indeed displaced by the competing ligand during the further 1 h of incubation. No such significant displacement was observed in control immunoprecipitates (Fig. 10).

DISCUSSION

The data presented here show that the interaction of bombesin with its specific receptor in the plasma membrane of Swiss 3T3 cells is specifically and intimately linked to the phosphorylation on tyrosine of an M_r 115,000 membrane protein. p115 tyrosine phosphorylation was detected in bombesin-stimulated intact cells by Western blotting analysis with P-Tyr antibodies. The extent of phosphorylation was dependent on both time of exposure to and dose of bombesin. With a fully mitogenic concentration, maximal p115 tyrosine phosphorylation was achieved after 30 min of stimulation at 37°C. It is noteworthy that this is the same time required for maximal binding of peptides of the bombesin family to their membrane receptors, as reported for these same cells by Zachary and Rozengurt (45). The dose dependence for p115 tyrosine phosphorylation was closely related to that observed for the mitogenic activity of bombesin on Swiss 3T3 cells (35). p115 tyrosine phosphorylation was a specific effect of bombesin on its target cells. In fact, no such effect was induced either by bombesin in cell types not bearing receptors for it or by different peptides and growth factors in Swiss 3T3 cells.

When Swiss 3T3 cells were extracted with a nonionic detergent in the presence of Zn^{2+}, a fraction of p115 was retained in the insoluble cell matrices and became phosphorylated in a bombesin-dependent manner when exposed to [γ-^{32}P]ATP. Phosphoamino acid analysis confirmed the presence of P-Tyr. The extent of phosphorylation was affected by exposure of the cell monolayers to bombesin prior to the extraction: the longer the exposure, the lower the extent of phosphorylation. This indicates a close relationship between bombesin interaction with its receptors and p115 phosphorylation and suggests a desensitization or downregulation process of p115 induced by bombesin in intact live cells.

When p115 was immunoprecipitated with P-Tyr antibodies from bombesin-stimulated cells and exposed to [γ-^{32}P]ATP and Mn^{2+}, it was phosphorylated on tyrosine by an associated bombesin-dependent kinase. Phosphoamino acid analysis also detected phosphoserine, but its content was not affected by the addition of bombesin to the phosphorylation buffer. As p115 was the major molecular species in immunoprecipitates from cells metabolically labeled with [^{35}S]methionine, and by analogy with the tyrosine kinases described so far, we interpret p115 phosphate incorporation as bombesin-dependent tyrosine autophosphorylation. The significant amount of phosphoserine noted in both phosphorylation assays may be ascribed to an unrelated serine kinase active in the permissive reaction conditions used. The occurrence of serine phosphorylation by unrelated enzymatic activities in the course of tyrosine kinase assays has been observed and discussed in detail for other proteins (7, 8).

The bombesin dependence of the tyrosine kinase activity associated with p115 was shown to be mediated by the presence of specific and saturable binding sites for bombesin in the same immunoprecipitate. The high affinity of the binding and the intimate link to p115 tyrosine phosphorylation were features shared with the bombesin receptor in the plasma membrane (compare reference 45 and the data presented above). Hence, the bombesin-dependent p115 tyrosine autophosphorylation in immunoprecipitates appears to be the in vitro reproduction of an early event in the interaction of bombesin with its target cells. The simplest interpretation of these data identifies p115 with a part of the transmembrane receptor for bombesin. This is also supported by the fact that the radiolabeled ligand is coprecipitated with p115 by P-Tyr antibodies from extracts of live intact cells exposed to the bombesin analog GRP. However, it is not possible to rule out that the saturable binding of the radiolabeled ligands is a function of association with a coprecipitating protein rather than p115. To identify the ligand-binding moiety of the receptor would require either cross-linking experiments or purification of the protein.

p115, as a membrane protein, is susceptible to extracellular proteolysis. In appropriately chosen experimental conditions, a lower-molecular-weight fragment—still recognizable by P-Tyr antibodies—was generated, and p115 immunolabeling disappeared. If this represented the truncated intracellular domain endowed with tyrosine kinase activity, a putative structure for the bombesin receptor complex in Swiss 3T3 cells would include a transmembrane protein of M_r 115,000 with an extracellular, trypsin-sensitive ligand-binding domain of approximate M_r 50,000 and an intracellular domain of M_r 65,000. The latter would be capable of phosphorylating itself on tyrosine after interaction of the whole molecule with peptides of the bombesin family.

Thus, the neuropeptide bombesin shares with other, better-known growth factors the property of inducing a receptor-associated tyrosine kinase activity in target cells. In the case of PDGF, EGF, and insulin, the kinase activity has been shown to be intrinsic to the receptor molecule, which then autophosphorylates on tyrosine after ligand binding (17, 19). Whether the bombesin receptor carries both the ligand-binding site and the catalytic domain on the same molecule, as suggested above, or whether it is a multisubunit assembly remains to be ascertained.

As with other mitogens, the interaction of bombesin with its membrane receptor elicits a number of early, receptor-linked responses in the target cell. Bombesin was previously reported to activate polyphosphoinositide turnover and protein kinase C (2). Here we show that it induces a receptor-associated tyrosine kinase activity. The relative significance of these events for the transduction of the mitogenic signal to the cell interior is still unknown, as is also true for most models studied so far. P-Tyr antibodies failed to detect major intracellular substrates for the kinase activity here described, in analogy with data reported for cells stimulated by PDGF (46).

However, the tyrosine phosphorylation of the bombesin receptor may be successfully exploited as a marker for tracing and characterizing the receptor molecule itself in other tissues. In particular, the relationship of the receptor described here with the one(s) responsible for the different physiological effects of bombesin deserves future study. In addition, the occurrence and the role of a tyrosine kinase related to the p115 protein should be investigated in human small cell lung carcinomas in which an autocrine circuit of growth stimulation has been suggested for bombesin (6).

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