Neuropeptide-stimulated tyrosine phosphorylation of specific components in Swiss 3T3 cells was investigated using monoclonal antibodies directed against the src transformation-associated substrates p125 focal adhesion kinase (FAK), a novel type of cytosolic tyrosine kinase, and p130. Treatment of Swiss 3T3 cells with the mitogenic peptides bombesin, vasopressin, and endothelin caused a striking increase in the tyrosine phosphorylation of p125FAK, as judged either by anti-phosphotyrosine (anti-Tyr(P)) Western blots of anti-p125FAK immunoprecipitates, or by anti-p125FAK immunoblots of anti-Tyr(P) immunoprecipitates. Bombesin-stimulated tyrosine phosphorylation of p125FAK was detectable within seconds and concentration-dependent (half-maximum effect of 0.3 μM). Neuropeptides also stimulated the tyrosine phosphorylation of a second component of M, 130,000, previously identified as the major p130 phosphotyrosyl protein in src-transformed cells. Bombesin stimulated p130 tyrosine phosphorylation with kinetics and concentration dependence similar to those observed for p125FAK. This is the first report to identify substrates for neuropeptide-stimulated tyrosine phosphorylation; the finding that one of these substrates is a tyrosine kinase suggests the existence of a novel signal transduction pathway in the action of mitogenic neuropeptides.

Tyrosine phosphorylation has recently been implicated in the action of neuropeptides that act as potential cellular growth factors (1–3) through G protein-coupled receptors (4–8). The neuropeptide growth factors bombesin, vasopressin, and the mouse endothelin VIC, stimulate tyrosine phosphorylation of multiple components in cultured Swiss 3T3 cells, including a broad band in the M, 110,000–130,000 range (4–6). None of the substrates for neuropeptide tyrosine phosphorylation have been identified, although they are unrelated to some known targets for receptor protein tyrosine kinases (9), including GTPase-activating protein, phosphatidylinositol 3'-kinase, and phospholipase C-γ (4, 10).

Non-receptor tyrosine kinases increase the phosphorylation of a distinct spectrum of cellular substrates compared with receptor tyrosine kinases (11–13). Tyrosine phosphorylation of multiple cellular proteins, including p125 and p130 components, is elevated in chicken embryo fibroblasts expressing activated variants of pp60src and the oncogene products of other avian sarcoma viruses (11–14). These transformation-associated substrates may play a role in the pathways that lead to transformation by pp60src and the oncogene products of other avian sarcoma viruses (11–14). Like p125FAK, p130 is a major tyrosine-phosphorylated protein in src-transformed fibroblasts and forms stable complexes with activated forms of pp60src (11, 13). The normal cellular function(s) of these proteins remain poorly understood.

In the present paper, we have used specific mAb raised against p125FAK and p130 (12) to investigate the possibility that these proteins could serve as targets for neuropeptide-stimulated tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Swiss 3T3 fibroblasts were maintained and propagated for experimental purposes as described (15).

Labeling with [35S]Methionine—Cells were washed twice with DMEM without methionine and then incubated for 16 h in 1 ml of DMEM containing [35S]methionine (20 μM, 50 μCi/ml).

Immunoprecipitations—Quiescent cultures of cells (1 × 10^6) were washed twice with DMEM, treated with peptide factors in 1 ml of this medium as indicated, and lysed at 4°C in 1 ml of a solution containing 10 mM Tris/HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na3V04, and 1% Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at 15,000 × g for 10 min and precleared by incubation with albumin-agarose for 1 h at 4°C. After removal of albumin-agarose by brief (10 s) centrifugation, the supernatants were transferred to fresh tubes for immunoprecipitation. The src-transformation-associated proteins p125FAK and p130 were immunoprecipitated for 3 h at 4°C with 1 μg/ml mAb 2A7 and mAb 4F4 (12), respectively. Phosphotyrosyl proteins were immunoprecipitated with the anti-phosphotyrosine (anti-Tyr(P)) mAb, 1G2 (Oncogene Science, Inc.) coupled to agarose (4, 5). The 2A7 and 4F4 mAb were precoupled to protein A-agarose and rabbit anti-mouse IgG prior to immunoprecipitation. Immunoprecipitates were washed three times with lysis buffer and further analyzed by Western blotting.

Western Blotting—Treatment of quiescent cultures of cells with factors, cell lysis, and immunoprecipitations were performed as described above. After SDS-PAGE, proteins were transferred to Immobilon membranes (4, 5). Membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 3–5 h with either the Py20 anti-Tyr(P) mAb or with mAb 2A7 as indicated in phosphate-buffered saline containing 0.05% Tween 20 and 1 μl/ml antibody. Immunoreactive bands were visualized using [125I]-labeled sheep anti-rabbit IgG. In extracts of 3T3 cells, we found that mAb 4F4 was not suitable for Western blotting.
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Materials—Bombesin, vasopressin, and albumin-agarose were obtained from Sigma. VIC was from Peninsula Laboratorios Inc. 2A7 and 4F4 mAbs were the generous gift of Thomas Parsons and Randy Vines, University of Virginia. Agarose-linked anti-Tyr(P) mAb was purchased from Oncogene Science Inc. Py20 anti-Tyr(P) mAb was from ICN. 35S-Labeled sheep anti-mouse immunoglobulin G (15 Ci/mmol) and [35S]methionine (1000 Ci/mmol) were from Amersham, U.K. Other reagents used were of the purest grade available.

RESULTS AND DISCUSSION
To investigate whether the recently identified tyrosine kinase p125FAK can serve as a substrate for neuropeptide-stimulated tyrosine phosphorylation, quiescent Swiss 3T3 cells were incubated with 10 nM bombesin, 20 nM vasopressin, or 10 nM VIC for 10 min, and lysates of the neuropeptide-treated cells were immunoprecipitated with mAb 2A7, which recognizes p125FAK (12, 14). The immunoprecipitates were analyzed by Western blotting with a specific anti-Tyr(P) mAb. Fig. 1A (left) shows that bombesin, vasopressin and VIC caused a striking increase in the tyrosine phosphorylation of a single anti-p125 immunoprecipitable band. This tyrosine-phosphorylated component exactly comigrated with the band obtained by immunoprecipitation of p125FAK from parallel cell lysates followed by immunoblot analysis using mAb 2A7 instead of anti-Tyr(P).

Further substantiate that neuropeptides stimulate tyrosine phosphorylation of p125FAK, anti-Tyr(P) immunoprecipitates derived from lysates of neuropeptide-treated cells were immunoblotted with anti-p125 mAb. Similar to the results shown in Fig. 1A, bombesin, vasopressin, and VIC caused a striking increase in the p125FAK immunoreactivity recovered by immunoprecipitation with anti-Tyr(P) (Fig. 1A, right).

Tyrosine phosphorylation of p125FAK was an extremely rapid consequence of addition of bombesin to Swiss 3T3 cells. An increase in phosphorylation as revealed by anti-Tyr(P) immunoblotting of anti-p125 immunoprecipitates was detected as early as 30 s after addition of the peptide, reached a maximum within 1 min, and remained relatively constant for up to 1 h (Fig. 1B). Bombesin-stimulated p125FAK phosphorylation was also concentration-dependent: half-maximum and maximum effects were obtained at 0.3 and 1 nM, respectively (Fig. 1C). Immunoblotting with mAb 2A7 of anti-p125 immunoprecipitates prepared in parallel with those used for analysis of the kinetics and concentration dependence of tyrosine phosphorylation (Fig. 1, B and C, lower gel) verified that similar amounts of p125FAK were recovered after different times and concentrations of bombesin treatment.

Fig. 1. Neuropeptides rapidly stimulate p125FAK tyrosine phosphorylation in intact Swiss 3T3 cells. A: left, quiescent Swiss 3T3 cells were incubated for 10 min at 37 °C with either 10 nM bombesin (B), 20 nM vasopressin (VP), 10 nM VIC, or an equivalent volume of solvent (—). Cells were then lysed, and lysates were incubated with mAb 2A7 for 3 h. Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of proteins to nitrocellulose and Western blotting with anti-Tyr(P). Right, cells were treated with neuropeptides and lysed, and lysates were then immunoprecipitated with anti-Tyr(P) mAb. Immunoprecipitates were analyzed by Western blotting with mAb 2A7. The position of p125FAK is indicated by an arrowhead. The positions of molecular weight markers (× 10^3) are shown on the left. The results shown are representative of at least three identical experiments. Other experimental details are described under "Experimental Procedures." B and C, cells were treated either with 10 nM bombesin for the times shown (panel B) or for 10 min with the indicated concentrations of bombesin (panel C), lysed, and immunoprecipitated with mAb 2A7. Immunoprecipitates were analyzed by Western blotting with either anti-Tyr(P) mAb (upper gel) or with mAb 2A7 (lower gel). The position of p125FAK is indicated with an arrowhead.

| Table 1 | Effect of pre-incubation with anti-Tyr(P) mAb on the recovery of p125FAK from lysates of control and bombesin-treated cells |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| Quiescent Swiss 3T3 cells were treated either in the absence (control) or presence of 10 nM bombesin for 10 min and lysed. The lysates were pre- incubated with anti-Tyr(P) mAb for 16 h, and subsequently the immunoprecipitates were collected and analyzed by Western blotting with mAb 2A7 (anti-Tyr(P) mAb). The supernatants of anti-Tyr(P) mAb immunoprecipitates were subsequently incubated with mAb 2A7 for another 6 h and then immunoblotted with mAb 2A7 (anti-Tyr(P) mAb — p125FAK). Lysates from parallel cells were incubated with mAb 2A7 for 6 h after incubating for 16 h with rabbit anti-mouse IgG coupled to protein A-agarose instead of anti-Tyr(P) mAb. Recovery of p125FAK from depleted and undepleted lysates was monitored by Western blotting using mAb 2A7. As shown in Table I, the recovery of immunoreactive p125FAK from lysates of bombesin-treated cells was strikingly decreased from 87 ± 11% to 22 ± 6% (n = 4) by prior removal of tyrosine-phosphorylated p125FAK. In contrast, preincubation of control lysates with anti-Tyr(P) mAb caused only a small decrease in the level of immunoreactive p125FAK (Table I). These conclusions were further supported by experiments in which lysates were prepared from control and bombesin-
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The relationship between the M, 130,000 band and a second src transformation-associated substrate, p130, was examined by immunoprecipitating lysates of bombesin-treated cells with a specific anti-p130 monoclonal antibody, mAb 4F4, followed by immunoblotting with anti-Tyr(P). As shown in Fig. 2A (lanes 3 and 4), bombesin markedly increased the phosphorylation content of a major anti-p130-immunoprecipitable band of M, 130,000. Furthermore, removal of p130 by incubating cell lysates with mAb 4F4, followed by anti-Tyr(P) immunoprecipitation and Western blotting, resulted in the depletion of a diffuse band which comigrated with the M, 130,000 phosphotyrosyl component present in anti-Tyr(P) and 4F4 immunoprecipitates (Fig. 2A, lanes 3–6). In contrast, immunodepletion of p130 did not reduce the level of a discrete M, 125,000 band (Fig. 2A, lanes 5 and 6), which comigrated with p125F-kinase immunoprecipitated with mAb 2A7 (Fig. 2A, lanes 7 and 8).

An increase in anti-phosphotyrosine immunoreactivity of p130 could be detected as early as 15 s after addition of bombesin, reaching a maximum after 1–2 min (Fig. 2B). Thereafter, p130 phosphorylation declined but remained above control unstimulated levels for up to 1 h. The effect of bombesin on p130 phosphorylation was concentration-dependent: half-maximal and maximum effects were obtained at 0.5 and 1 nM, respectively (Fig. 2C). Vasopressin and VIC also markedly increased the tyrosine phosphorylation of p130 within seconds and at nanomolar concentrations (results not shown). The concentration dependence of neuropeptide-stimulated p125F-kinase and p130 tyrosine phosphorylation closely paralleled the concentration dependence for the effect of neuropeptides on the reinitiation of DNA synthesis (16–18).

Several reports have recently described neuropeptide stimulation of tyrosine phosphorylation in a variety of cell types, but neither the tyrosine kinase(s) nor the substrates involved were identified (4–8). The findings presented here demonstrate for the first time that the src transformation-associated substrates p125F-kinase and p130 are targets for neuropeptide-stimulated tyrosine phosphorylation in Swiss 3T3 cells. The identification of p125F-kinase as a novel cytosolic tyrosine kinase with a unique structural architecture (14) suggests that this protein may function as a downstream element in a neuropeptide-stimulated tyrosine kinase pathway. Further experimental work will be necessary to elucidate whether p130 serves as a substrate for p125F-kinase and whether the increase in anti-phosphotyrosine phosphorylation of p125F-kinase by neuropeptides is the result of autophosphorylation or the action of a more proximal kinase. Regardless of the precise mechanism(s), our results identify a previously unrecognized signal transduction link in the action of neuropeptide growth factors which act through G protein-coupled receptors.

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treated cells that had been labeled with [35S]methionine. Incubation of these lysates with mAb 2A7 resulted in the immunoprecipitation of a number of bands, including a major band which comigrated with p125F-kinase. Similar to the results shown in Table I, recovery of [35S]methionine-labeled p125F-kinase from bombesin-treated but not control lysates was decreased by 70 ± 3% (n = 3) after preincubation with anti-Tyr(P) mAb. These results strongly suggest that bombesin stimulates tyrosine phosphorylation of p125F-kinase to a high stoichiometry.

To establish that p125F-kinase is a constituent of the broad M, 110,000–130,000 phosphotyrosyl band stimulated by neuropeptides, p125F-kinase was removed from cell lysates prepared from bombesin-treated cells by preclearing with three consecutive incubations with mAb 2A7. Anti-Tyr(P) immunoblots of anti-Tyr(P) immunoprecipitates prepared from p125-immunodepleted lysates revealed a marked reduction in anti-Tyr(P) immunoreactivity of a band that exactly comigrated with p125F-kinase. In contrast, immunodepletion of p125F-kinase did not affect bombesin-stimulated tyrosine phosphorylation of other components including a diffuse M, 130,000 band (results not shown), which comigrated with a component present in anti-Tyr(P) Western blots of undepleted anti-Tyr(P) immunoprecipitates (Fig. 2A, lanes 1 and 2).

Several reports have recently described neuropeptide stimulation of tyrosine phosphorylation in a variety of cell types, but neither the tyrosine kinase(s) nor the substrates involved were identified (4–8). The findings presented here demonstrate for the first time that the src transformation-associated substrates p125F-kinase and p130 are targets for neuropeptide-stimulated tyrosine phosphorylation in Swiss 3T3 cells. The identification of p125F-kinase as a novel cytosolic tyrosine kinase with a unique structural architecture (14) suggests that this protein may function as a downstream element in a neuropeptide-stimulated tyrosine kinase pathway. Further experimental work will be necessary to elucidate whether p130 serves as a substrate for p125F-kinase and whether the increase in anti-phosphotyrosine phosphorylation of p125F-kinase by neuropeptides is the result of autophosphorylation or the action of a more proximal kinase. Regardless of the precise mechanism(s), our results identify a previously unrecognized signal transduction link in the action of neuropeptide growth factors which act through G protein-coupled receptors.

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FIG. 2. Bombesin rapidly stimulates p130 tyrosine phosphorylation in intact Swiss 3T3 cells. A, cells were treated either in the absence (lanes 1, 3, 5, and 7) or in the presence of 10 nM bombesin (lanes 2, 4, 6, and 8) for 10 min and lysed. Some cell lysates were precleared of p130 by incubating for 3 h three times consecutively with mAb 4F4 using fresh antibody each time. These lysates were subsequently immunoprecipitated with anti-Tyr(P) mAb and analyzed by Western blotting with anti-Tyr(P) (lanes 5 and 6). Lysates prepared from parallel cultures were immunoprecipitated either with anti Tyr(P) mAb (lanes 1 and 2), mAb 4F4 (lanes 3 and 4), or mAb 2A7 (lanes 7 and 8), and the immunoprecipitates were then analyzed by Western blotting with anti-Tyr(P). The positions of p130 and p125F-kinase and the phosphotyrosyl proteins, p90, p81, and p75 (4), are indicated by arrowheads. B, C, cells were incubated either with 10 nM bombesin for different times (panel B) or for 10 min with different concentrations of bombesin (panel C). The cells were then lysed, immunoprecipitated with mAb 4F4, and analyzed by Western blotting with anti-Tyr(P) as described. The position of p130 is indicated by an arrowhead.
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