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Mitogenic Signals and Transforming Potential of Nyk, a Newly Identified Neural Cell Adhesion Molecule-Related Receptor Tyrosine Kinase

LEI LING AND HSING-JIEN KUNG*

Department of Molecular Biology and Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4960

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Nyk/Mer is a recently identified receptor tyrosine kinase with neural cell adhesion molecule-like structure (two immunoglobulin G-like domains and two fibronectin III-like domains) in its extracellular region and belongs to the Ufo/Axl family of receptors. The ligand for Nyk/Mer is presently unknown, as are the signal transduction pathways mediated by this receptor. We constructed and expressed a chimeric receptor (Fms-Nyk) composed of the extracellular domain of the human colony-stimulating factor 1 receptor (Fms) and the transmembrane and cytoplasmic domains of human Nyk/Mer in NIH 3T3 fibroblasts in order to investigate the mitogenic signaling and biochemical properties of Nyk/Mer. Colony-stimulating factor 1 stimulation of the Fms-Nyk chimeric receptor in transfected NIH 3T3 fibroblasts leads to a transformed phenotype and generates a proliferative response in the absence of other growth factors. We show that phospholipase Cγ, phosphatidylinositol 3-kinase/p70 S6 kinase, Shc, Grb2, Raf-1, and mitogen-activated protein kinase are downstream components of the Nyk/Mer signal transduction pathways. In addition, Nyk/Mer weakly activates p90^{rsk}, while stress-activated protein kinase, Ras GTPase-activating protein (GAP), and GAP-associated p62 and p190 proteins are not activated or tyrosine phosphorylated by Nyk/Mer. An analysis comparing the Nyk/Mer signal cascade with that of the epidermal growth factor receptor indicates substrate preferences by these two receptors. Our results provide a detailed description of the Nyk/Mer signaling pathways. Given the structural similarity between the Ufo/Axl family receptors, some of the information may also be applied to other members of this receptor tyrosine kinase family.

Growth and differentiation signals are often initiated by the interactions of growth factors with specific receptors (36). For many growth factors, the receptors are tyrosine kinases. Largely on the basis of the structural motifs in the extracellular domain, the receptor tyrosine kinases can be subdivided into nine families (27). Among those, the most extensively studied are the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) families. The newest family, Ufo/Axl, is characterized by its neural cell adhesion molecule (NCAM)-related extracellular domains (i.e., two immunoglobulin G [IgG]-like domains juxtaposed with the two fibronectin III-like domains). *ufo*/*axl* was originally identified as a transforming gene derived from chronic myeloproliferative disorders (41) and chronic myelogenous leukemias (67). Ark was identified as the mouse homolog of Ufo/Axl (28, 74). As is NCAM, the NCAM domain of Ark is able to induce cell aggregation by homophilic binding, which in turn activates the kinase activity (2). In addition, recent studies have shown that soluble ligands such as Gas6 can bind and trigger the kinase activity of Ufo/Axl (88, 93). Thus, there appear to be multiple mechanisms through which this family of receptor tyrosine kinases can be activated.

In the course of our analysis of potential tyrosine kinases involved in the growth of glioblastomas, we have isolated a cDNA clone from a human glioblastoma expression library which corresponds to a new member of the Ufo/Axl family. We designate this receptor tyrosine kinase Nyk (or NCAM-related tyrosine kinase). The putative chicken homolog of this gene, c-*eyk*, was identified by Jia et al. as the homolog of a sarcoma-inducing oncogene of a retrovirus (42, 43). Graham et al. recently reported the sequence and the tissue distribution of the same receptor, which they referred to as Mer (for tyrosine kinase of monocytes and epithelial and reproductive tissues) (33). Other than the above-mentioned studies, no information is available concerning the signaling pathway or the ligand for this receptor. In this paper, we report the initial characterization of the transforming potential and the signaling pathways mediated by Nyk. We use the more generic name Nyk or Nyk/Mer to reflect its relatedness to NCAM and wider tissue distribution.

A strategy that has been used successfully for the study of receptors with unidentified ligands involves the construction of chimeric receptor molecules, in which the extracellular domain is replaced by the ligand-binding region of another receptor tyrosine kinase with known ligands. Previous studies of such hybrid molecules have amply demonstrated the fidelity of this approach and that the intracellular domains are the primary determinants of the signaling pathway (21, 53–55, 80, 81). As a first step to define the functional and biochemical features of the Nyk/Mer receptor tyrosine kinase, we have analyzed its mitogenic properties and signal transduction pathways in murine NIH 3T3 fibroblasts. To this end, we constructed a chimeric receptor, designated Fms-Nyk, consisting of the ligand-binding domain of the human colony-stimulating factor 1 receptor (CSF-1R or Fms) (17) and the transmembrane, tyrosine kinase, and C-terminal domains of human Nyk. Since NIH 3T3 cells do not have endogenous Fms (84), this provides a background-free environment to study the transforming potential and signaling pathways of Nyk. We show here that

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Case Western Reserve University, School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106-4960. Phone: (216) 368-6655. Fax: (216) 368-3055.

fms-nyk is a potent transforming gene for NIH 3T3 cells. Both the kinase activity and the transformation ability are CSF-1 dependent. We have analyzed the Nyk-induced major signaling pathways and compared the results with those for EGFR. There are clearly substrate preferences for Nyk. Nyk efficiently activates the phosphatidylinositol 3-kinase (PI3K) pathway, the Shc/Grb2/Raf-1/mitogen-activated protein kinase (MAPK) pathway, and the phospholipase Cγ (PLCγ) pathway. It has little or no effect on Ras GTPase-activating protein (GAP) and stress-activated protein kinase (SAPK) pathways. To our knowledge, this is the first comprehensive dissection of the signal transduction pathways of Nyk or the Ufo/Axl receptor tyrosine kinase family as a whole.

MATERIALS AND METHODS

Reagents. Recombinant human CSF-1 was purchased from Cellular Products Inc. (Buffalo, N.Y.). Human EGF was from Collaborative Research Inc. The antibody specific for Fms was obtained from Oncogene Science (Uniondale, N.Y.). The antiphosphotyrosine, anti-EGFR, anti-PLCγ, anti-GAP, anti-PI3K p85 subunit, anti-p70 S6 kinase, anti-Raf, anti-Shc, anti-Grb2, and anti-p90^{rsk} antibodies were purchased from Upstate Biotechnology Inc. Anti-MAPK and anti-SAPK antibodies were from Santa Cruz Biotechnology, Inc. The anti-Nyk antibody was obtained by immunizing New Zealand White rabbits with a bacterially expressed glutathione *S*-transferase (GST) fusion protein containing amino acids 835 to 930 of the human Nyk receptor.

Construction of the Fms-Nyk cDNA. The DNA encoding the extracellular domain of human Fms was kindly provided by Mark Tycocinski (Institute of Pathology, Case Western Reserve University). The extracellular domain of Fms (amino acids 1 to 512) was fused to the transmembrane and catalytic domains of human Nyk (amino acids 502 to 999) by PCR. Briefly, the extracellular domain of human Fms was amplified with a 5′ primer derived from Bluescript KS+ (Stratagene) and a 3′ primer encoding the antisense sequences of human Fms amino acids 506 to 512 and human Nyk amino acids 502 to 506 (5′ AAA GAT GAT GAG CAC CTC ATC CGG GGG ATG CGT GTG 3′; the underlined sequences are from Nyk). The transmembrane and intracellular regions of Nyk were amplified with a 5′ primer encoding the sense sequence of human Fms amino acids 508 to 512 and human Nyk amino acids 502 to 508 (5′ CAT CCC CCG GAT GAG GTG CTC ATC ATC TTT GGC TGC 3′; the underlined sequences are from Nyk) and a 3′ primer from Bluescript KS+. The two PCR fragments were annealed and reamplified. The resulting molecule was verified by DNA sequencing (U.S. Biochemical). The chimeric Fms-Nyk molecule was placed under the control of the Moloney murine leukemia virus long terminal repeat promoter in the mammalian expression vector AFVXM (47). The Fms-Nyk chimera terminates 103 nucleotides downstream of the Nyk translation termination codon.

Cell lines and culture conditions. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO). The Fms-Nyk.3T3 cell lines (6×10^3 chimeric receptors per cell) were generated by transfection of NIH 3T3 cells with the AFVXM/Fms-Nyk retroviral vector and a hygromycin-resistant plasmid, PY3 (6). After selection in 0.2 mg of hygromycin (Calbiochem) per ml for 3 weeks, the stably transfected cells were subcloned and screened for expression of the chimeric Fms-Nyk by Western blotting (immunoblotting) with a polyclonal antiserum against the C terminus of Nyk. The EGFR.3T3 cell line (10^6 EGFR per cell) is an NIH 3T3 derivative overexpressing human EGFR as described previously (3).

Cell proliferation assays. For serum-free culture, NIH 3T3 and Fms-Nyk.3T3 cells were seeded in triplicate at 2×10^4 cells per 35-mm-diameter dish in DMEM with 10% FBS and after 24 h transferred to DMEM supplemented with 5 ng of sodium selenite (Sigma) per ml, 10 μg of transferrin (Sigma) per ml, 0.5% bovine serum albumin (Boehringer Mannheim), and 0.05 mM 2-mercaptoethanol in the presence or absence of 2,000 U of human recombinant CSF-1 per ml. Viable cell numbers were determined daily by trypan blue exclusion.

[^{3}H]thymidine incorporation assays. Confluent cell monolayers on 96-well culture plates were grown in serum-free DMEM for 24 h. DNA synthesis was stimulated by various concentrations (0, 100, 500, 1,000, 2,000, and 10,000 U/ml) of human CSF-1 for 18 h, and then 4 h of pulse-labeling with 2 μCi of [*methyl*-^{3}H]thymidine (Amersham) per ml was carried out. Cells were subjected to five freeze-thaw cycles, and incorporated radioactivity was determined with a 1205 Betaplate scintillation counter (Wallac).

Transformation soft agar assays. To investigate whether activation of the Fms-Nyk receptor in mouse fibroblasts can also release contact inhibition of their growth, NIH 3T3 and Fms-Nyk.3T3 cells were plated in duplicate at a density of 5×10^3 cells per 35-mm-diameter dish in the presence or absence of 1,000 U of recombinant human CSF-1 per ml in DMEM–10% FBS–0.3% Noble agar (Difco) over a bottom layer of DMEM–10% FBC–0.5% Noble agar. Photographs were taken after 2 to 3 weeks of growth at 37°C.

Immunoprecipitation and immunoblotting analyses. Prior to stimulation with growth factors, NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were starved in serum-free DMEM for 24 to 48 h. After treatment at 37°C with human CSF-1 (2,000 U/ml) and human EGF (100 ng/ml) for various times, cells were washed in phosphate-buffered saline and lysed in lysis buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl [pH 7.3], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 10 μg of pepstatin per ml). Cell lysates were clarified by centrifugation at 13,000 × *g* for 10 min. Immunoprecipitations were performed for 2 to 6 h at 4°C with the appropriate antiserum and then bound to protein A- or G-coupled Sepharose (Zymed). The immunoprecipitates were washed three times with the lysis buffer, resuspended with double-strength electrophoresis sample buffer (2× ESB) (0.125 M Tris-HCl [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue), boiled, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). After transfer of the proteins onto polyvinylidene difluoride Immobilon-P membrane (Millipore), the membrane was blocked with 3% bovine serum albumin in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) for 1 h and incubated first with the appropriate primary antibody for 2 h and then with goat anti-rabbit or goat anti-mouse alkaline phosphatase conjugate (Bio-Rad). The blot was subsequently developed in Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) substrates (Sigma).

PI3K assays. For measuring receptor-associated PI3K activity in vivo, the cells were stimulated with ligand CSF-1 (2,000 U/ml), EGF (100 ng/ml), or PDGF (100 ng/ml) for 10 min or left unstimulated. Next, the cells were lysed with NP-40 lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 100 μM sodium orthovanadate, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride) (3, 94). The lysates were incubated with antiphosphotyrosine antibody and protein A-Sepharose beads. The immunoprecipitates were washed extensively and incubated with 50 μl of kinase reaction mix containing 10 mM Tris (pH 7.5), 0.1 M NaCl, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 20 mM $MgCl_2$, 0.2 mg of phosphatidylinositol (Sigma) per ml, and 10 μCi of [γ-^{32}P]ATP (Amersham). The reaction was allowed to proceed for 20 min at room temperature and stopped by adding 100 μl of 1 M HCl and then 200 μl of methanol-chloroform (1:1). The sample was mixed and spun for 15 min. The lower chloroform phase was dried, resuspended, and applied to thin-layer chromatography plates (Merck). After 5 to 6 h of chromatography in a tank equilibrated with 1-propanol and acetic acid (1-propanol–2 M acetic acid, 13:7), the thin-layer chromatography plates were subjected to autoradiography at −70°C overnight.

p70 S6 kinase, p90^{rsk}, Raf-1, and SAPK assays. After stimulation with ligand, the cells were lysed in lysis buffer as described above. The p70 S6 kinase and p90^{rsk} were immunoprecipitated with the respective antibodies. After being washed three times in lysis buffer, the immune complexes were incubated in 50 μl of kinase reaction mix containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 15 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP, and 20 μg of substrate peptide RRLSSLRA (5, 70). To terminate the reaction, aliquots were removed, spotted on Whatman P81 phosphocellulose paper, and washed extensively in phosphoric acid and acetone. The radioactivity retained on the paper was counted by a scintillation counter. For the inhibition of p70 S6 kinase activity, cells were pretreated with 20 ng of rapamycin (Calbiochem) per ml for 30 min at 37°C prior to addition of ligand CSF-1. The kinase activity is expressed as counts per minute incorporated into the substrate peptide RRLSSLRA after subtraction of the background counts.

For Raf-1 kinase assays, immunoprecipitates were prepared with 1 μg of anti-Raf-1 antibody per sample. The kinase reaction was performed as described above except that 1 μg of kinase-inactive (Lys-Ala) GST-MEK fusion protein (Upstate Biotechnology Inc.) was used as a substrate. To terminate the reaction, 2× ESB was added. The reaction mixture was then resolved on an SDS–10% PAGE gel and exposed to X-ray film.

For SAPK assays, immunoprecipitates were prepared with 1 μg of anti-SAPK antibody. The substrate for SAPK assays was 1 μg of GST–c-Jun (N terminus) (48) for each reaction. The reaction mixture was resolved on an SDS–10% PAGE gel and exposed to X-ray film.

RESULTS

Developing cell lines expressing the chimeric Fms-Nyk molecule. To investigate the functions of Nyk/Mer, we constructed a chimeric receptor, Fms-Nyk, fusing the human Fms extracellular domain to the Nyk transmembrane and cytoplasmic domains (Fig. 1A). The chimera was placed in a Moloney murine leukemia virus-based retroviral vector (47) and expressed in NIH 3T3 mouse fibroblasts. We chose the NIH 3T3 cell line for our initial studies because of the wealth of information available for its signal transduction pathways and its usefulness in assessing transformation potential. NIH 3T3 cells do not

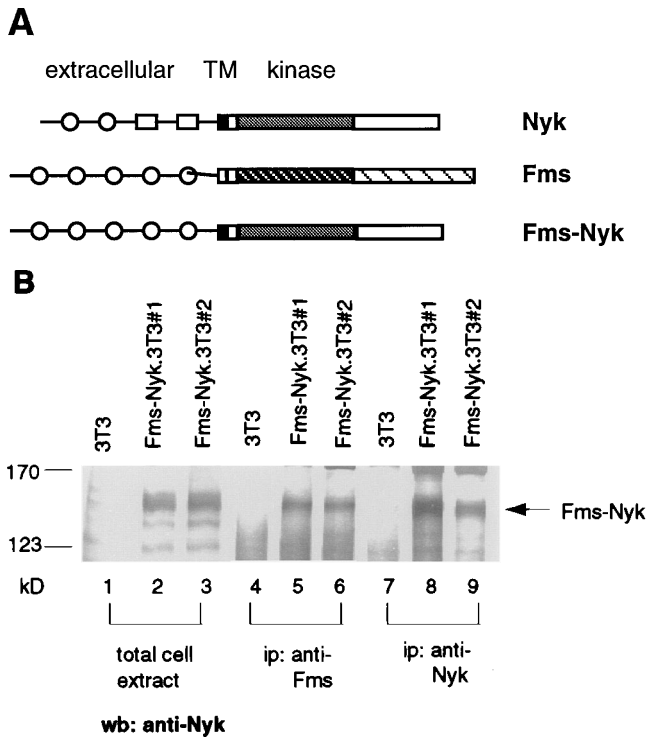

FIG. 1. Structure and expression of the Fms-Nyk chimera. (A) Schematic diagrams of Nyk, Fms, and the chimeric receptor Fms-Nyk. Fms-Nyk contains the extracellular region of Fms and the transmembrane (TM) and intracellular regions of Nyk. The tyrosine kinase domain is indicated. The circles and rectangles in the extracellular region represent, respectively, the IgG-like domains and fibronectin III-like domains. Details of the construction are described in Materials and Methods. (B) Stable expression of the chimeric Fms-Nyk receptor on NIH 3T3 cells. Parental NIH 3T3 and transfected Fms-Nyk.3T3 cells (clones Fms-Nyk.3T3#1 and Fms-Nyk.3T3#2) were lysed and subjected to immunoprecipitation (ip) with the monoclonal antibody against the extracellular domain of Fms (lanes 4 to 6) or a polyclonal antibody to the C terminus of Nyk (lanes 7 to 9). The Western blotting (wb) was performed by using a polyclonal antibody to the C terminus of Nyk. Total cell extracts were also analyzed in parallel (lanes 1 to 3). The 150-kDa chimeric protein Fms-Nyk is shown by an arrow.

contain endogenous Fms and do not respond to treatment with human CSF-1 (84). In addition, we have previously established a 3T3 cell line (EGFR.3T3) that overexpresses human EGFR and is transformable by ligands to EGFR (3). This serves as a useful control to compare and contrast the signal transduction pathways utilized by the two different families of receptors.

The expression of the chimeric receptor Fms-Nyk was directly demonstrated by Western blotting with GST-human Nyk (C terminus)-reactive polyclonal antisera. As shown in Fig. 1B, no Nyk-related protein can be detected in parental NIH 3T3 cells while the two independent Fms-Nyk.3T3 transfectants express a 150-kDa protein, detectable by anti-Nyk antibody (Fig. 1B, lanes 1 to 3). That the 150-kDa polypeptide is the chimeric Fms-Nyk molecule was further confirmed by immunoprecipitation with either the anti-human Fms antibody (Fig. 1B, lanes 4 to 6) or the anti-Nyk antibody (lanes 7 to 9), followed by blotting with anti-Nyk antibody. At the same time, no signal was detected from parental NIH 3T3 cells. The observed molecular mass is higher than the calculated value (100 kDa), presumably because of glycosylation.

In the above-described and all subsequent experiments, two independent Fms-Nyk.3T3 cell lines were used and the results from these two lines were similar. For the purpose of clarity, only the results from one cell line are shown for the following experiments.

Activation of Fms-Nyk chimera by CSF-1. To demonstrate the functional integrity of the Nyk/Mer domains of the chimeric receptor, we analyzed ligand-induced tyrosine phosphorylation of Fms-Nyk in the Fms-Nyk.3T3 cell lines. The serum-starved cells were stimulated with increasing concentrations of CSF-1 (0 to 10,000 U/ml) for 10 min (Fig. 2A) or with 2,000 U of CSF-1 per ml for different periods of time (0 min to 2 h) (Fig. 2B), and equivalent amounts of total cell lysates were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody to examine the overall tyrosine phosphorylation pattern. As shown in Fig. 2A, among others, two proteins with the sizes of 150 and 42 kDa became prominently phosphorylated on tyrosine residues in a CSF-1-dosage-dependent manner. These two proteins comigrate with Fms-Nyk and MAPK, respectively (see below). Figure 2B shows the kinetics of chimera phosphorylation in response to CSF-1 stimulation. The tyrosine phosphorylation of the Fms-Nyk chimera reached maximum levels 10 min after CSF-1 stimulation and was reduced after 30 min. As a comparison, cell lysates from EGFR.3T3 cells, a cell line overexpressing human EGFR (3), was also analyzed (Fig. 2B, lanes 7 and 8). EGF induction for 10 min gives a phosphorylation pattern distinct from that of Fms-Nyk, although several common bands are also detected. The lower panels of Fig. 2A and B show that the amount of Fms-Nyk remains constant during stimulation of the cells. Figure 2C shows that the CSF-1 effect is via the Fms-Nyk chimeric receptor, as the parental NIH 3T3 cells do not respond to CSF-1 stimulation. These results, taken together, suggest that the chimeric Fms-Nyk is kinase active and inducible by ligand CSF-1.

Mitogenic properties and transforming potential of the chimeric Fms-Nyk receptor. Having demonstrated that the chimeric Fms-Nyk is biochemically active, we asked whether it is mitogenic and exhibits anchorage-independent transforming potential. Elevation of tyrosine phosphorylation levels for receptor tyrosine kinases is necessary but not always sufficient to trigger a proliferative signal (29, 85). To examine the capacity of the chimeric Fms-Nyk molecule to transmit a proliferative signal, the effect of human CSF-1 on the growth properties of Fms-Nyk.3T3 cells was analyzed.

As shown in Fig. 3A, the Fms-Nyk.3T3 cells cultured in serum-free DMEM grow slowly in the absence of CSF-1. However, when supplemented with 2,000 U of CSF-1 per ml, these cells are capable of continuous proliferation. The parental NIH 3T3 cells grow very slowly in the presence and in the absence of CSF-1, indicating that the effect is not due to CSF-1 per se. In addition, we measured the incorporation of [*methyl*-^{3}H]thymidine into the DNA of cells exposed to increasing concentrations of CSF-1 (Fig. 3B). Within the range of CSF-1 concentrations studied (0 to 10,000 U/ml), no significant changes in thymidine incorporation were observed to occur in parental, untransfected cells. By contrast, CSF-1 stimulated DNA synthesis in Fms-Nyk.3T3 cells, in proportion to the concentration of CSF-1 applied. Both clones of Fms-Nyk.3T3 cells form anchorage-independent soft agar colonies in the presence of CSF-1, while the mock-transfected parental NIH 3T3 cells do not, even after 4 to 5 weeks (Fig. 3C).

These results demonstrate that Fms-Nyk is a receptor of strong mitogenic and transforming potential. Our finding echoes the original discovery of *eyk*, the putative chicken homolog of *nyk*, as a sarcoma-inducing oncogene in a retrovirus (42, 43).

Signal transduction pathways mediated by Fms-Nyk. Several studies have identified various SH2-containing proteins as the immediate receptor tyrosine kinase substrates (1, 44, 69). These substrates have helped define several major, and in some cases overlapping, pathways. They include PLCγ, PI3K/

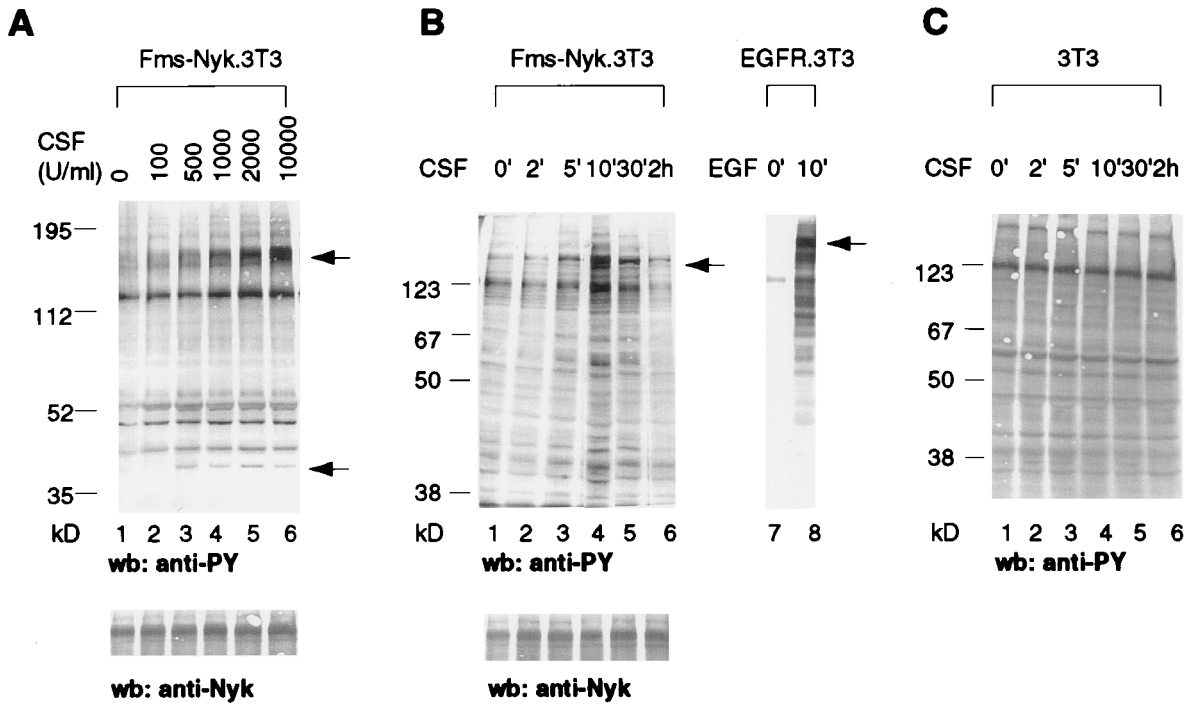

FIG. 2. Ligand-induced tyrosine phosphorylation of Fms-Nyk. (A) Fms-Nyk.3T3 cells were stimulated with the indicated concentrations of human CSF-1 for 10 min. Total cell lysates were subjected to Western blotting (wb) with antiphosphotyrosine (anti-PY) antibodies (upper panel) or anti-Nyk antibodies (lower panel). The positions of the 150-kDa Fms-Nyk protein and the 42-kDa MAPK are indicated by arrows. (B) Fms-Nyk.3T3 cells were incubated with 2,000 U of human CSF-1 per ml for the indicated times. Total cell lysates were subjected to Western blotting with antiphosphotyrosine antibodies (upper panel) or anti-Nyk antibodies (lower panel). In parallel (lanes 7 and 8), extracts from EGFR.3T3 cells which have been stimulated with 100 ng of EGF per ml were also analyzed. The positions at which the 150-kDa Fms-Nyk protein and the 170-kDa EGFR migrate are indicated with arrows. (C) Parental NIH 3T3 cells were stimulated with CSF-1 as described for panel B and Western blotted with antiphosphotyrosine antibodies.

p70 S6 kinase, GAP, and Shc/Grb2/Raf/MEK/MAPK pathways (44, 61). To investigate the signaling pathways mediated by the Nyk/Mer receptor tyrosine kinase, we first examined the phosphorylation of substrates involved in the above-mentioned four major pathways in Fms-Nyk.3T3 cells. In parallel studies, we analyzed the phosphorylation of these substrates by the EGFR tyrosine kinase in EGFR.3T3 cells.

Tyrosine phosphorylation of PLCγ by Fms-Nyk. PLCγ is an isoform of phospholipase C which couples receptor tyrosine kinases to the hydrolysis of phosphatidylinositol-4,5-bisphosphate and generates diacylglycerol and inositol-1,4,5-triphosphate as the second messengers. PLCγ is phosphorylated on tyrosine residues by various activated receptors, including PDGFR and EGFR (59, 66, 95), but not by activated CSF-1R (22). Thus, there is a certain degree of selectivity in the utilization of PLCγ as a substrate. To determine the potential role of PLCγ in Nyk-mediated signal transduction, Fms-Nyk.3T3 cells were stimulated with CSF-1 and PLCγ immunoprecipitates were analyzed by Western blotting with antiphosphotyrosine antibodies. As shown in Fig. 4, the 140-kDa PLCγ is tyrosine phosphorylated in response to the activation of Fms-Nyk, with the maximum phosphorylation occurring at 10 min after ligand treatment. The controls show that PLCγ does not respond to CSF-1 in the parental NIH 3T3 cells but does respond to EGF in EGFR.3T3 cells. The PLCγ levels were essentially equivalent in all lanes, as demonstrated in the lower panels of Fig. 4.

Activation of PI3K/p70 S6 kinase by Fms-Nyk. PI3K is an enzyme that phosphorylates the inositol ring of phosphatidylinositol at the D-3 position. PI3K has been characterized as a heterodimer with a regulatory p85 and a catalytic p110 subunit (11, 39). Most receptor tyrosine kinases activate the PI3K pathway through recruitment of the PI3K activity with the fraction of phosphotyrosine-containing proteins (16, 73, 77, 94). To define the possible interaction of PI3K with Nyk/Mer kinase, extracts from Fms-Nyk.3T3 cells were immunoprecipitated with antiphosphotyrosine antibodies and analyzed for antiphosphotyrosine antibody-associated PI3K activity by kinase reaction. Figure 5A shows that PI3K is activated by the Fms-Nyk chimera in response to CSF-1. This activation of PI3K is at least 10-fold stronger than is stimulation of EGFR.3T3 cells by EGF, and it reaches a level comparable to that induced by PDGF treatment. Expression of PI3K in those cells was confirmed by parallel immunoblotting with anti-PI3K antiserum (Fig. 5A, lower panels). In a separate experiment, the PI3K was found to be associated directly with the activated Nyk receptor (data not shown).

Recent studies revealed that PI3K is the upstream activator of the 70-kDa ribosomal S6 kinase (p70 S6 kinase), a serine/threonine kinase capable of phosphorylating the 40S ribosomal protein S6 (15, 46, 90). To test the possibility that p70 S6 kinase is activated by the Nyk/Mer signaling pathway, we conducted the p70 S6 kinase assay using a synthetic peptide derived from S6 protein as a substrate (70). As shown in Fig. 5B, upon CSF-1 stimulation, the p70 S6 kinase was activated in Fms-Nyk.3T3 cells to a degree much higher than the activation by EGF in EGFR.3T3 cells. In addition, this activation can be inhibited by pretreating the cells for 30 min with 20 ng of rapamycin per ml, which is a specific inhibitor of p70 S6 kinase (72). In control NIH 3T3 cells, p70 S6 kinase was not activated by CSF-1.

These findings, taken together, suggest that an active Nyk

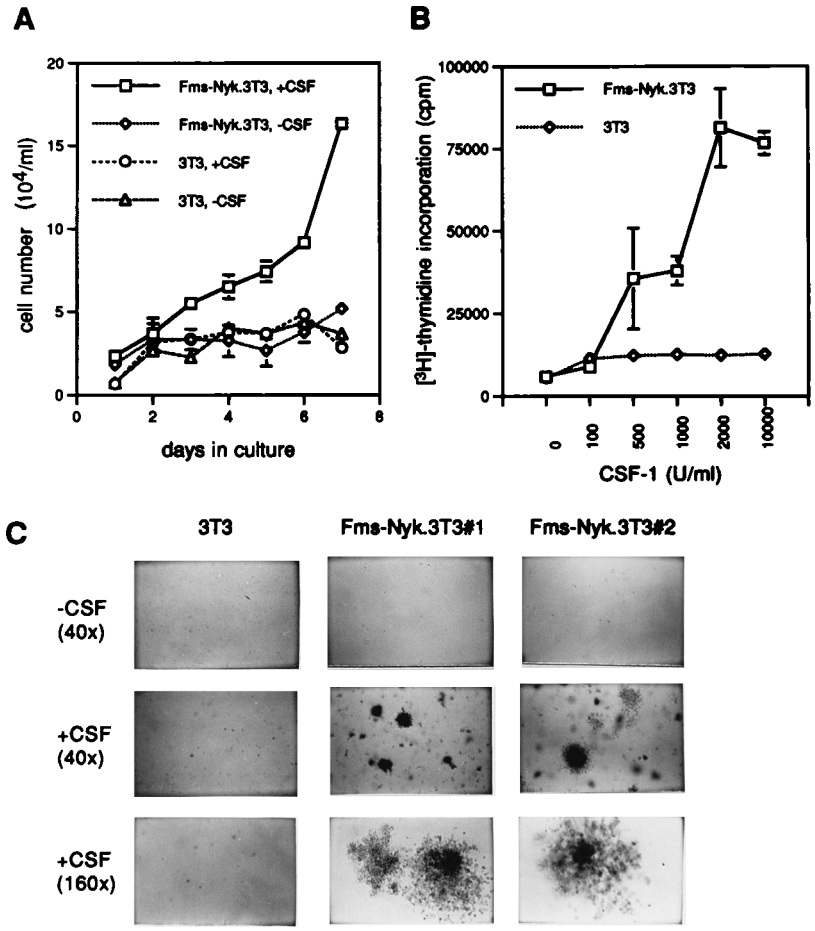

FIG. 3. Mitogenic properties of Fms-Nyk. (A) CSF-1-dependent growth of Fms-Nyk.3T3 cells in serum-free medium. NIH 3T3 and Fms-Nyk.3T3 cells were cultured with or without 2,000 U of CSF-1 per ml as described in Materials and Methods. Viable cells from triplicate cultures were counted daily. The standard error at each time point is indicated by a vertical bar ($n = 3$). (B) CSF-1-dependent DNA synthesis of Fms-Nyk.3T3 cells. NIH 3T3 and Fms-Nyk.3T3 cells were seeded in triplicate in 96-well plates. [^{3}H]thymidine incorporation was measured after stimulation of the cells with CSF-1 at the indicated concentrations. The standard error at each concentration point is indicated by a vertical bar ($n = 3$). (C) CSF-1-induced anchorage-independent growth of Fms-Nyk.3T3 cells in soft agar. Fms-Nyk.3T3 cells (clones Fms-Nyk.3T3#1 and Fms-Nyk.3T3#2) and mock-transfected NIH 3T3 cells were grown in soft agar with or without 1,000 U of CSF-1 per ml. Colonies were photographed after 3 weeks. The magnifications (×40 and ×160) of the photographs are indicated.

kinase is able to associate with and activate PI3K with an efficiency comparable to that of the PDGFR, which activates PI3K at least 10-fold more efficiently than the EGFR. This activation is accompanied by the activation of the p70 S6 kinase.

Lack of tyrosine phosphorylation of GAP and associated proteins by Fms-Nyk. GAP can stimulate the GTPase activity of p21^{ras} and convert p21^{ras} from the active GTP-bound form to the inactive GDP-bound form (63). GAP has been shown to associate with and become phosphorylated by certain tyrosine kinase receptors such as EGFR (7, 26). The tyrosine phosphorylation of GAP is also accompanied by the association of two other tyrosine-phosphorylated proteins, p62 and p190 (26, 83, 96). To assess the possible role of GAP and associated proteins in the Nyk/Mer signal transduction pathway, lysates of CSF-1-stimulated Fms-Nyk.3T3 cells were immunoprecipitated with GAP-reactive antisera and blotted with antiphosphotyrosine antibody. Little increase in tyrosine phosphorylation of GAP and associated p62 was detected in Fms-Nyk.3T3 cells after CSF-1 stimulation (Fig. 6). The treatment of EGFR.3T3 cells with EGF, on the other hand, results in increases in the amounts of tyrosine-phosphorylated GAP and, most notably,

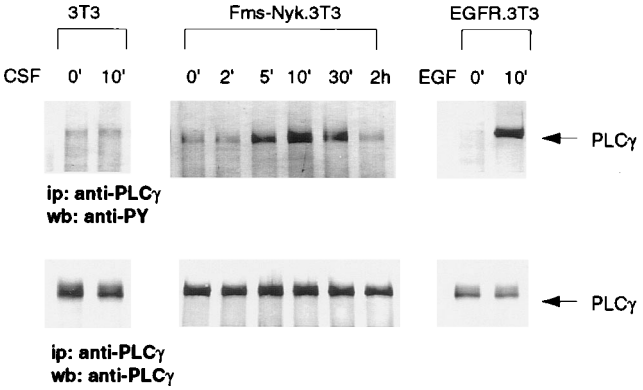

FIG. 4. Tyrosine phosphorylation of PLCγ by Fms-Nyk. NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for the indicated times. Anti-PLCγ immunoprecipitates (ip) were divided for Western blot (wb) analyses with antiphosphotyrosine antibodies (upper panels) or anti-PLCγ antibodies (lower panels). The 140-kDa PLCγ is shown by an arrow.

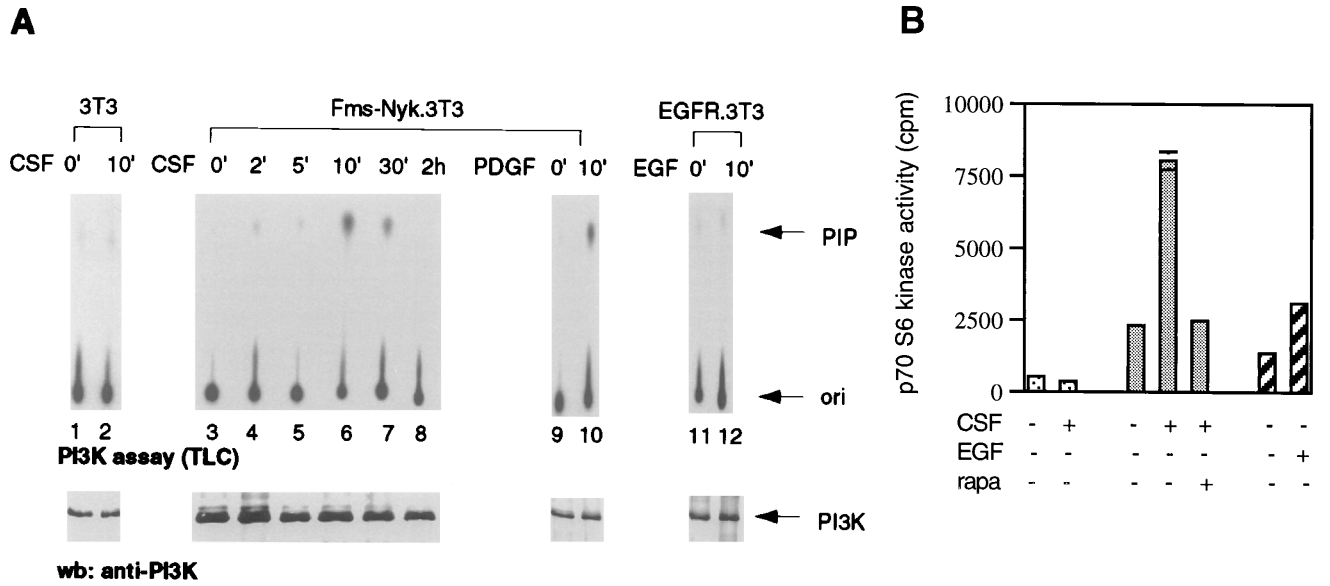

FIG. 5. Activation of PI3K and p70 S6 kinase by Fms-Nyk. (A) Total cell extracts from cells treated with CSF-1 (2,000 U/ml), PDGF (100 ng/ml), or EGF (100 ng/ml) were immunoprecipitated with antiphosphotyrosine antibodies. The immunoprecipitates were assayed for PI3K activity as described in Materials and Methods and analyzed by thin layer chromatography (TLC). The origin (ori) and the position where phosphatidylinositol 3-phosphate (PIP) migrates are indicated. For the lower panels, the total cell extracts were separated by SDS-PAGE and Western blotted (wb) with a rabbit polyclonal antibody recognizing the p85 subunit of PI3K. (B) NIH 3T3 (spotted bars), Fms-Nyk.3T3 (shaded bars), and EGFR.3T3 (striped bars) cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for 10 min or left unstimulated. The p70 S6 kinase activity was assessed by incubating the anti-p70 S6 kinase immunoprecipitates with [γ-^{32}P]ATP and substrate peptide RRLSSLRA. The products of the reaction were spotted on phosphocellulose filters and washed extensively. The radioactivity incorporated into the substrate was quantitated with a liquid scitillation counter. For the rapamycin (rapa) inhibition experiment, the Fms-Nyk.3T3 cells were preincubated with 20 μg of rapamycin per ml at 37°C for 30 min before CSF-1 stimulation. The standard error at each point is indicated by a vertical bar (n = 3). In most cases, the standard errors are too small to be visualized.

the tyrosine-phosphorylated p62. On the basis of these data, GAP activation does not seem to be a significant pathway for Fms-Nyk.

Activation of the Shc/Grb2/Raf-1/MAPK/p90^{rsk} pathway by Fms-Nyk. The MAPK pathway has been well described (4, 8). In most cases, this pathway begins with the phosphorylation of Shc and the association of Grb2 (23, 71). Grb2 then translocates Sos to the cell surface, where it catalyzes the formation of Ras-GTP (24, 56). The binding of Ras-GTP to Raf-1 kinase activates a series of serine/threonine kinases in the order of Raf-1, MEK, and MAPK (4, 8, 52, 58).

We first examined the phosphorylation of Shc to determine whether Nyk/Mer can couple to this signaling cascade. Three isoforms of Shc, p46^{shc}, p52^{shc}, and p66^{shc}, have been reported (64, 71). Shc proteins were immunoprecipitated with anti-Shc antibody and blotted with antiphosphotyrosine antibody. As shown in Fig. 7A, Shc proteins isolated from CSF-1-stimulated Fms-Nyk.3T3 cells show a slightly higher phosphotyrosine content than do Shc proteins in nonstimulated cells. As expected, EGF induces a stronger phosphorylation of Shc, with the phosphorylation of the p52^{shc} and p66^{shc} isoforms more apparent. The comigration of p46^{shc} with the precipitating IgG heavy chain obscures the detection of the former. A consequence of Shc phosphorylation is the recruitment of Grb2 to the receptor-Shc complex. Cell extracts immunoprecipitated with anti-Shc antibody were blotted with anti-Grb2 antibody (Fig. 7B). It is evident that Grb2 coprecipitates with Shc in CSF-1-treated Fms-Nyk.3T3 cells and EGF-treated EGFR.3T3 cells, but not in untreated NIH 3T3 control cells. Grb2 is also associated with the activated receptor Fms-Nyk and EGFR by immunoprecipitating the receptors and Western blotting with anti-Grb2 antibody (Fig. 7C). Figure 7D shows that total cell lysates containing equivalent amounts of Shc proteins were used in Fig. 7A, B, and C. The observations of CSF-1-dependent Shc and Grb2 recruitment by the chimeric receptor Fms-Nyk suggest that the Ras pathway participates in Nyk-mediated signal transduction.

We then analyzed the activation of the two downstream

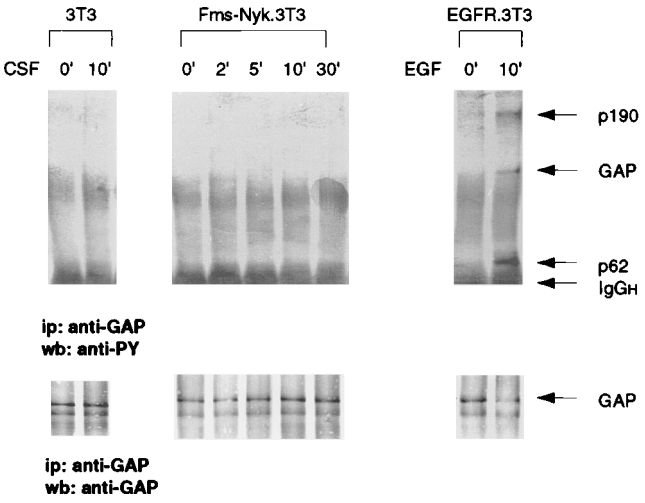

FIG. 6. Lack of tyrosine phosphorylation of GAP by Fms-Nyk. NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for the indicated times or left unstimulated. Anti-GAP immunoprecipitates (ip) were divided for Western blot (wb) analyses with antiphosphotyrosine antibodies (anti-PY) (upper panels) or anti-GAP antibodies (lower panels). The positions at which GAP, p190, p62, and IgG heavy chain (IgGH) migrate are indicated by arrows.

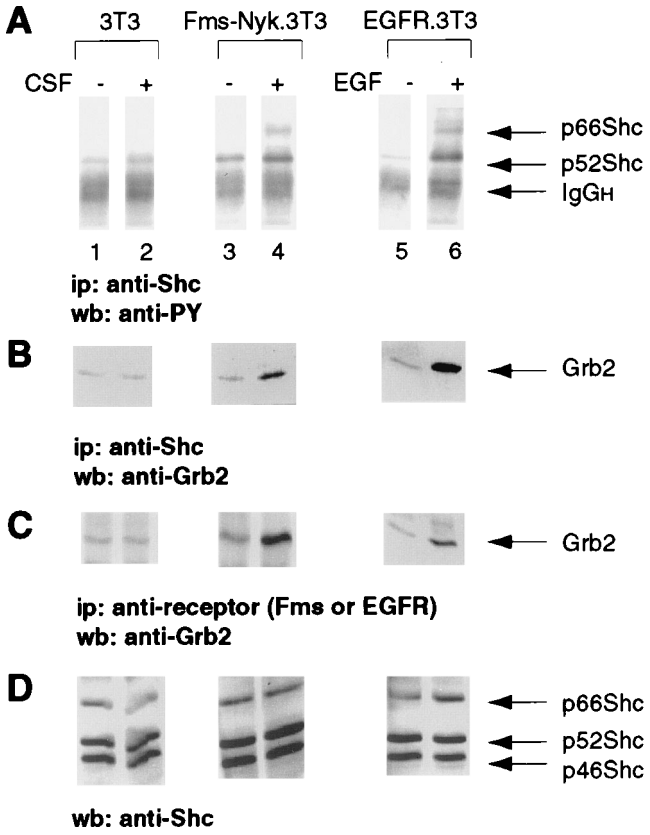

FIG. 7. Ligand-dependent recruitment of Shc and Grb2 by Fms-Nyk. NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for 10 min or left unstimulated. (A) Tyrosine phosphorylation of Shc proteins. Equal amounts of the total cell extracts were immunoprecipitated (ip) with anti-Shc polyclonal antibody, analyzed on an SDS–7.5% PAGE gel, and Western blotted (wb) with antiphosphotyrosine (anti-PY) antibodies. The positions for $p52^{shc}$, $p66^{shc}$, and IgG heavy chain (IgGH) are indicated. (B) Shc-associated Grb2. Anti-Shc immunoprecipitates were separated on a 12% polyacrylamide gel and blotted with a monoclonal anti-Grb2 antibody. (C) Receptor-associated Grb2. Cell extracts from 3T3 and Fms-Nyk.3T3 cells were immunoprecipitated with anti-Fms antibody, and extracts from EGFR.3T3 cells were precipitated with anti-EGFR antibody. These antireceptor immunoprecipitates were separated on a 12% polyacrylamide gel and blotted with anti-Grb2 antibody. (D) Western blots of Shc. Aliquots (50 μg) of total cell extracts used for panels A, B, and C were subjected to direct Western blotting with the anti-Shc antibody. The positions of Shc proteins (p66, p52, and p46 isoforms) are indicated by arrows.

serine/threonine kinases, Raf-1 and MAPK, in the Ras pathway. Activated Raf-1 phosphorylates MEK, a tyrosine and serine dual kinase (51). To assess the Raf-1 activity, a kinase-inactive GST-MEK (see Materials and Methods) was used as a substrate to avoid the complication due to MEK autophosphorylation. The Raf-1 protein was immunoprecipitated, and its kinase activity was assessed by the incorporation of [γ-^{32}P]ATP into the kinase-inactive GST-MEK. As shown in Fig. 8, Raf-1 kinase is activated in Fms-Nyk.3T3 cells in response to CSF-1 stimulation.

The activation of MAPK is characterized by phosphorylation at both tyrosine and threonine residues and its consequential shift in electrophoretic mobility (76, 82). We used an anti-MAPK antibody which recognizes both the p44 and the p42 isoforms of MAPK in immunoprecipitation and Western blotting. As shown in Fig. 9A, tyrosine-phosphorylated p42 and p44 MAPK were readily detectable in Fms-Nyk.3T3 cells. The activation peaked at 10 min after CSF-1 stimulation with a

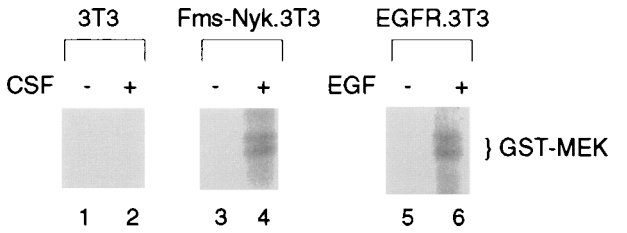

FIG. 8. Raf-1 activation in Fms-Nyk.3T3 cells. NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for 10 min or left unstimulated. Raf kinase activity was assessed by incubating the anti-Raf-1 immunoprecipitates with [γ-^{32}P]ATP and kinase-inactive GST-MEK as a substrate. The products of the reaction were separated by SDS–10% PAGE and exposed to X-ray film.

level comparable to that seen in EGF-treated EGFR.3T3 cells. This finding is echoed by the electrophoretic shift of the activated p42 MAPK (Fig. 9B). The antibody used in this study for Western blotting recognized only the p42 MAPK. As shown in Fig. 9B, in Fms-Nyk.3T3 cells, CSF-1 stimulation resulted in the dose-dependent mobility shift of the p42 MAPK, reaching a maximum of about 30%.

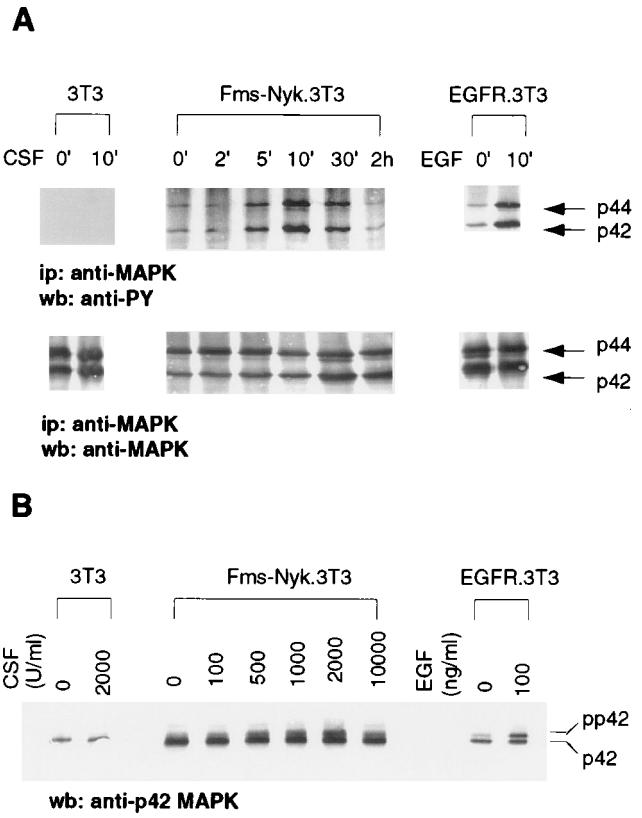

FIG. 9. MAPK activation by Fms-Nyk. (A) NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for the indicated times. Anti-MAPK immunoprecipitates (ip) were divided for Western blot (wb) analyses with antiphosphotyrosine antibodies (anti-PY) (upper panels) or anti-MAPK antibodies (lower panels). The MAPKs were separated by SDS–7.5% PAGE (1.5-mm-thick gels). The p44 and p42 MAPK isoforms are indicated by arrows. (B) NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with the indicated concentrations of human CSF-1 or EGF for 10 min. Total cell lysates were separated by SDS–10% PAGE (0.75-mm-thick gels) and subjected to Western blotting with anti-p42 MAPK antibody. The positions of the unphosphorylated p42 MAPK (p42) and the phosphorylated, supershifted p42 MAPK (pp42) are indicated.

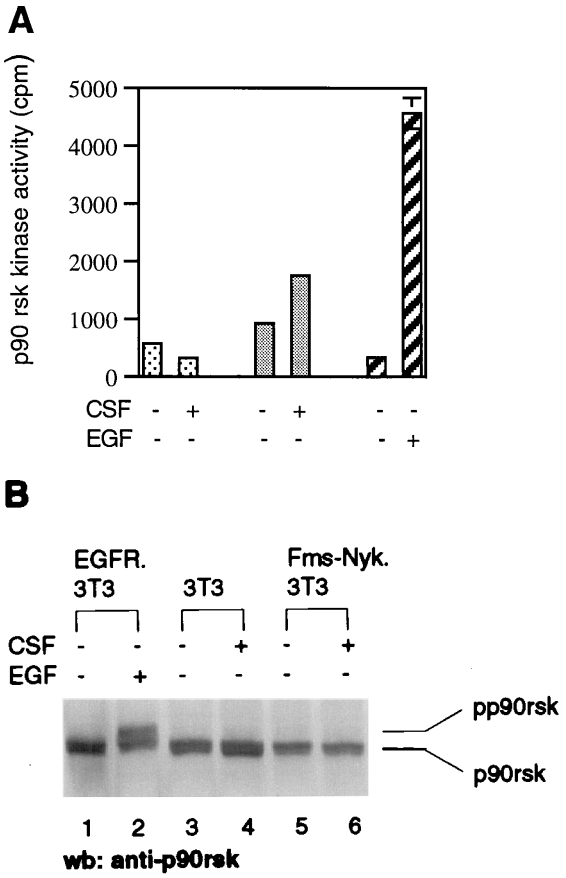

FIG. 10. $p90^{rsk}$ activation by Fms-Nyk. (A) NIH 3T3 (spotted bars), Fms-Nyk.3T3 (shaded bars), and EGFR.3T3 (striped bars) cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for 10 min or left unstimulated. $p90^{rsk}$ kinase activity was assessed by incubating the anti-$p90^{rsk}$ kinase immunoprecipitates with [γ-^{32}P]ATP and substrate peptide RRLSSLRA. The products of the reaction were spotted on phosphocellulose filters and washed extensively. The radioactivity incorporated into the substrate was quantitated with a liquid scintillation counter. The standard error at each point is indicated by a vertical bar (n = 3). In most cases, the standard errors are too small to be visualized. (B) Cells were stimulated as described for panel A. A 50-μg amount of total cell extracts was analyzed by SDS-PAGE and Western blotted (wb) with anti-$p90^{rsk}$ antibody. The positions of the unphosphorylated $p90^{rsk}$ kinase ($p90^{rsk}$) and the phosphorylated, supershifted $p90^{rsk}$ kinase ($pp90^{rsk}$) are indicated.

The immediate downstream substrates for activated MAPK are transcriptional factors such as Elk (32, 40) and, in some cases, the serine/threonine kinase 90-kDa ribosomal S6 kinase ($p90^{rsk}$) (25, 30, 45, 89). $p90^{rsk}$ activates the transcriptional factor serum response factor (SRF), which in turn activates c-fos (12, 14, 75, 92). $p90^{rsk}$ activation correlates with its ability to phosphorylate 40S ribosome S6 protein in vitro and its retarded mobility on SDS-PAGE (13, 14). Thus, we analyzed these two parameters of $p90^{rsk}$ kinase following activation of Fms-Nyk. In the positive-control EGFR.3T3 cells, treatment with EGF resulted in a high level of incorporation of radioactivity into the S6 substrate peptide (Fig. 10A) and a significant mobility shift of the $p90^{rsk}$ protein (Fig. 10B). By contrast, in CSF-1-stimulated Fms-Nyk.3T3 cells, only weak elevation of $p90^{rsk}$ kinase activity and little or no mobility shift were observed (Fig. 10). This identifies another divergence of the EGFR and Nyk pathways and raises the possibility that MAPK may not be the sole in vivo activator of $p90^{rsk}$.

The lack of activation of SAPK kinase by Fms-Nyk. SAPK (also called JNK) is a distant relative of the MAPK group that is activated by dual phosphorylation at Thr and Tyr by a number of stress factors, including UV, tumor necrosis factor alpha, and anisomycin (9, 20, 38, 48, 78, 86). Recent studies have shown that SAPK is activated by MEK kinase (MEKK) and SEK, a pathway parallel to that of Raf-1 and MEK (57, 97). In turn, the activated SAPK is responsible for the direct phosphorylation of c-Jun at the N-terminal Ser-63 and Ser-73, thereby activating c-Jun (79). We compared the activations of SAPK in Fms-Nyk.3T3 cells and EGFR.3T3 cells. SAPK activity was assayed by immunoprecipitating SAPK first and then performing a kinase reaction with a GST–c-Jun (N terminus) fusion protein as the substrate (48). As shown in Fig. 11, stimulation of Fms-Nyk.3T3 cells resulted in little or no activation of SAPK, while EGF activated SAPK significantly. Anisomycin is a strong inducer of SAPK activity (9) and serves as a positive control here.

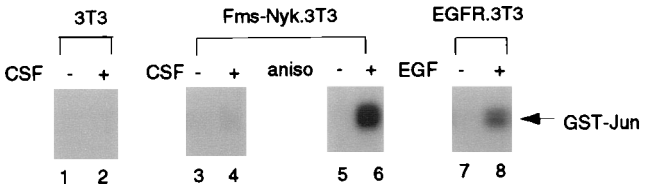

FIG. 11. Lack of SAPK activation by Fms-Nyk. NIH 3T3, Fms-Nyk.3T3, and EGFR.3T3 cells were stimulated with 2,000 U of human CSF-1 per ml or 100 ng of EGF per ml for 10 min or left unstimulated. As a positive control, Fms-Nyk.3T3 cells were incubated with 10 μg of anisomycin (aniso) per ml at 37°C for 30 min. SAPK kinase activity was assessed by incubating the anti-SAPK immunoprecipitates with [γ-^{32}P]ATP and bacterially expressed GST–c-Jun (N terminus) as a substrate. The products of the reaction were separated by SDS–10% PAGE and exposed to X-ray film.

Summary of the Nyk-mediated signals. Figure 12 summarizes the signal transduction pathways analyzed in this study. As will be discussed further below, Nyk and EGFR have several signaling pathways in common, but there are clear differences in the relative strengths and utilizations of substrates by these two receptors.

DISCUSSION

The Ufo/Axl receptor tyrosine kinase family now has at least three members with various names: Ufo/Axl/Ark/Tyro 7, Tyro 3/Sky/Rse/Brt/Tif, and Nyk/Mer/Eyk/Tyro 12 (18, 28, 31, 33, 41–43, 49, 50, 60, 67, 68, 74). They are characterized by a common NCAM-like motif present in the extracellular do-

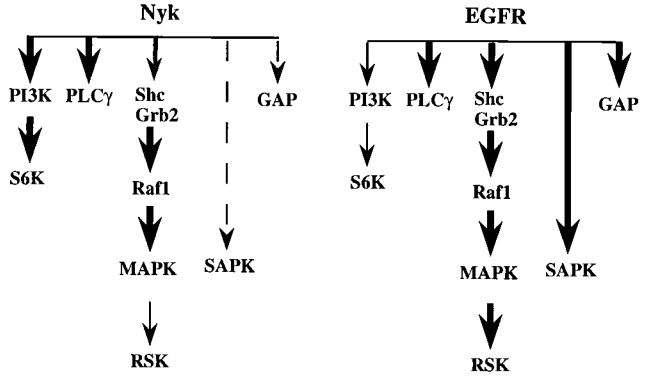

FIG. 12. Diagram of signal transduction pathways of Nyk compared with those of EGFR. Thick arrows, strong activation; thin arrows, weak activation; dotted arrows, activation undetectable.

main, a single membrane-spanning domain, and a cytoplasmic tyrosine kinase domain. The presence of an NCAM-like motif suggests that this family of kinases may signal through direct cell-cell contact. Evidence supporting such a view has been obtained for Ark, the mouse homolog of Ufo/Axl (2). On the other hand, recent studies by Stitt et al. (88) and by Varnum et al. (93) revealed the existence of soluble ligands for Ufo/Axl and Tyro 3/Sky, indicating that the regulation of Ufo/Axl family receptors may be complex and depends on the environmental context. The ligand for Ufo/Axl is Gas6, and that for Tyro 3/Sky is protein S; both are regulated by vitamin K modification (88, 93). The ligand for Nyk/Mer remains unidentified. While the identification of ligands for this family of receptors begins to shed light on the extracellular signaling process, little is known about the intracellular signaling pathways. Several studies have provided evidence for autophosphorylation of these receptors (2, 62, 67, 91), but few delved into the characterization of the downstream phosphorylation signals.

Chimeric constructs have often been used to study signal transduction pathways for orphan receptors (21, 53–55, 80, 81). While this approach may not provide a physiologically relevant environment, the general fidelity of the intracellular signaling is usually preserved. With Nyk, this approach offers special advantages since it provides a clean picture of the intracellular signaling without the complications due to cell-cell interaction or a possible autocrine loop. The data presented here should form a baseline for future understanding of how different extracellular signals may influence the intracellular activity of Nyk. Our construct bears the extracellular domain of human Fms and the transmembrane and intracellular domains of Nyk/Mer. The extracellular domain of Fms carries IgG-like motifs, but not fibronectin III-like motifs. Using human CSF-1 as a ligand, we found that Fms-Nyk is a potent mitogen and induces anchorage-independent growth in NIH 3T3 cells. These data are consistent with *eyk*, the putative chicken homolog of *nyk*, being originally identified as an oncogene of a sarcoma virus (42, 43). v-Eyk, the viral Eyk with constitutively active kinase activity, also assumes a chimeric form with the viral envelope protein gp37 fused to the Eyk intracellular domain (43). The fact that *ufo*/*axl* was also identified as an NIH 3T3-transforming gene (41, 65) strongly suggests that this family of receptors plays a critical role in mitogenic signaling.

To understand the signals engaged by Nyk, we examined the effects of an activated Fms-Nyk chimera on a number of well-recognized signaling intermediates using EGFR-induced signals as a reference point. In 3T3 cells, overexpression and activation of EGFR (3) or Fms-Nyk (this study) (Fig. 3C) result in anchorage-independent growth with similar cellular morphologies in soft agar. Intracellularly, Nyk and EGFR mediate their respective signals through several common signaling intermediates; however, distinctions have also been identified. As summarized in Fig. 12, EGFR induces strong tyrosine phosphorylation of PLCγ, Shc, GAP, and GAP-associated p62 and p190 proteins but weak activation of PI3K. Indeed, the weak activation of PI3K by EGFR was thought to occur via cross talk with the ErbB3 receptor (87). By contrast, Nyk does not induce phosphorylation of GAP and GAP-associated p62 and p190 proteins but is a potent activator of PI3K. The ability of Nyk to activate PI3K may be related to the presence of a PI3K SH2-binding consensus sequence (YMXM) (34, 37) encompassing amino acids 830 to 833 of Nyk. The activation of PI3K by Nyk is further supported by the heightened activity of p70 S6 kinase, a serine/threonine kinase believed to be downstream from PI3K (15). Rapamycin treatment abolishes Nyk-activated p70 S6 kinase activity, attesting to the specificity of the assay (72, 90). These data suggest that the PI3K pathway may play a more significant role in Nyk-induced than in EGFR-induced mitogenic signals.

Aside from PI3K, PLCγ and Shc are also immediate substrates for Nyk. The Shc/Grb2/MAPK pathway is induced by Nyk with a potency similar to that of EGFR. In cells treated with EGF or other ligands, the activated MAPK subsequently induces the activation of another serine/threonine kinase, $p90^{rsk}$, which translocates into the nucleus to activate SRF and hence activates c-*fos* transcription (12, 14, 75, 89). Here, Nyk's response again diverges from that of EGFR such that $p90^{rsk}$ is not significantly activated by Nyk, despite the high level of activated MAPK. This suggests that either Nyk activates a phosphatase that rapidly down-modulates $p90^{rsk}$ or the phosphorylation of $p90^{rsk}$ in vivo involves activation of additional factors absent in a Nyk-induced state.

The MAPK family has now been extended to include SAPK and p38 MAPK (10, 19, 35). SAPK is likely to be the enzyme responsible for the activation of c-Jun (20, 79). Recently, Yan et al. have shown that SAPK is activated principally by a number of stress-related factors via the MEKK/SEK pathway (97). In some cell types SAPK is also activated by EGF or TPA (65, 78). Our data show that SAPK is not a target of Nyk activation. The lack of SAPK activation and the weak activation of $p90^{rsk}$ are consistent with our findings that the Jun/Fos or AP-1 activity is not strongly induced by Nyk (data not shown). These observations reveal that Jun/Fos activation may not be a universal indicator of cell growth.

In summary, this report attempts to delineate the intracellular signaling process of Nyk/Mer. Nyk/Mer induces the phosphorylation and activation of PLCγ, PI3K/p70 S6 kinase, and Shc/Grb2/MAPK pathways, but not SAPK and GAP. Nyk is mitogenic and induces anchorage-independent transformation of the rodent cell line NIH 3T3. Our data provide a framework to understand the detailed mechanisms by which the Nyk/Mer receptor tyrosine kinase family transmits mitogenic signals.

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