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Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity

(cell division/nuclear localization/DNA binding)

HIDEO KIMURA

The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92186-5800

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ABSTRACT **Schwannoma-derived growth factor (SDGF) is a mitogen and neurotrophic protein which belongs to the epidermal growth factor (EGF) family. There are two basic amino acid clusters in the SDGF molecule which are homologous to the nuclear targeting signal of the simian virus 40-encoded large tumor antigen. Mutational analysis of these clusters showed that they function as nuclear targeting signals, and a gel retardation assay showed that SDGF binds to A+T-rich DNA sequences. Both the wild-type SDGF and a mutant defective in the nuclear targeting signals activate the immediate early genes NGFI-A and c-*fos*. The wild-type SDGF is a mitogen for Swiss mouse 3T3 fibroblasts, but the mutant defective in the nuclear targeting signals is not mitogenic. Moreover, wild-type SDGF potentiates [^{3}H]thymidine incorporation in NIH mouse 3T3 cells bearing an EGF receptor defective in the kinase domain, whereas the mutant SDGF does not stimulate DNA synthesis. These results suggest that transport into the nucleus is required for SDGF to induce a mitogenic response.**

After binding and receptor activation, most receptor–growth factor complexes are thought to be internalized, transported to lysosomes, and degraded (1, 2). Recently, however, examples have emerged where growth factors exert their effect directly in the nucleus. Exogenously applied platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) accumulate in the nucleus of target cells (3, 4), and their nuclear targeting signals have been identified (5–7). bFGF regulates gene transcription in a cell-free system (8). These findings indicate that growth factors may function within the nucleus. Schwannoma-derived growth factor (SDGF) was initially isolated from a schwannoma cell line as a mitogen for astrocytes and fibroblasts (9) and is neurotrophic (10). The following data show that the transport of SDGF into the nucleus is required for it to induce its mitogenic activity.

MATERIALS AND METHODS

Mutant genes were constructed by PCR. Each mutant has 27 bases encoding a 9-amino acid epitope (YPYDVPDYA) recognized by mouse monoclonal antibody 12CA5 (11). Transfections were done as described (9), and for staining, cells were fixed with 3% formaldehyde and treated with 0.5% Triton X-100. After 1 hr of incubation with antibody 12CA5, cells were incubated with fluorescently labeled goat anti-mouse antibody.

Cell fractionation (12), Western blot analysis (13), and [^{3}H]thymidine incorporation (9) were done as described. For gel retardation assays, a ^{32}P-labeled oligonucleotide consisting of a 15-base random oligonucleotide flanked by defined terminal ends of 15 bases each was incubated with 0.3 μg of glutathione *S*-transferase fusion protein at room temperature for 20 min (14). The retarded DNA was excised, eluted, and extracted with phenol/chloroform. PCR amplification was carried out and the resultant fragments were cloned and sequenced (15). Competition binding assays were performed as described (16).

To assay c-*fos* and nerve growth factor I-A (NGFI-A) induction, Swiss 3T3 cells were serum starved for 2 days, and RNA was extracted (17) 30 min after the addition of SDGF and subjected to Northern blot analysis.

RESULTS AND DISCUSSION

Since SDGF has two basic amino acid clusters which are homologous to the nuclear targeting signal of the simian virus 40 (SV40)-encoded large tumor antigen (18–20), the roles of the secretory signal sequence and the nuclear localization sequences of SDGF were determined by mutational analysis. These experiments were carried out by transfecting COS cells (SV40-transformed monkey cells) with an expression vector containing modified SDGF DNA, and the intracellular localization of SDGF was determined by two methods. Because SDGF is extensively modified by proteolytic cleavage of prepro-SDGF (9), the distribution and processing of SDGF was followed by introducing 27 bases encoding a 9-amino acid epitope (YPYDVPDYA) at the 3′ end of the SDGF coding region. This sequence is recognized by monoclonal antibody 12CA5 (11). Since secreted SDGF consists of at least two major forms, a long form (amino acids 97–243) and a short form (amino acids 97–175) (9), two variants of the proteins were constructed for mutational analysis (Fig. 1*a*). Both long and short forms of the precursor were found in the cytoplasm and the nucleus (Fig. 1*b*, lanes 1–4, and Fig. 2*a*). The proteins recognized by the anti-epitope monoclonal antibody were also recognized on Western blots by two rabbit antisera raised against fusion proteins (data not shown). To determine whether the deletion of the secretory signal peptide leads to nuclear localization, both the long and short forms of SDGF defective in their signal peptides were constructed. Both short and long forms of the secretory signal mutants were detected mainly in the nucleus (Fig. 1*b*, lanes 5–8, and Fig. 2*b*). These results show that the inhibition of secretion leads to enhanced nuclear accumulation. SDGF is also found in the nucleus of the JS1 cell line from which it was isolated (data not presented).

To determine whether the two basic sequences in SDGF function as nuclear targeting signals, they were mutagenized, leaving other regions unchanged (Fig. 1*f*) (22, 23). When amino acids 130–134 (RKKKK) were changed to neutral amino acids (ILTIL) (M2 mutant, Fig. 1*f*) the short form of the SDGF precursor localized primarily to the cytoplasm

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Abbreviations: SDGF, schwannoma-derived growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor.

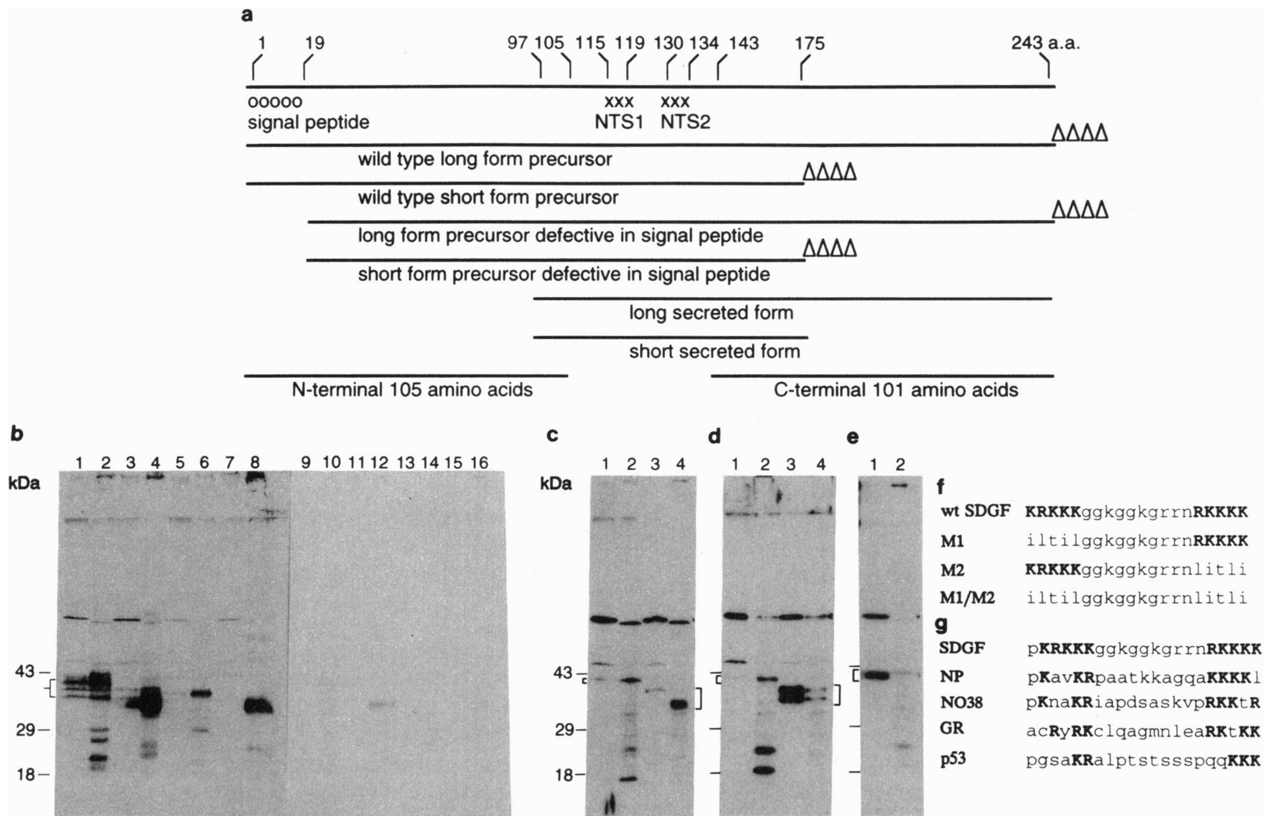

FIG. 1. Subcellular localization of SDGF in transiently transfected COS cells. (*a*) Schematic diagram of various mutants of SDGF and the mutated regions used to make fusion proteins. ΔΔΔΔ indicates the position of the attached epitope for 12CA5. NTS1 and NTS2 are the nuclear targeting signals. (*b*) Cytoplasmic (lanes 1, 3, 5, 7, 9, 11, 13, and 15) and nuclear (lanes 2, 4, 6, 8, 10, 12, 14, and 16) fractions from COS cells transfected with the wild-type long form of SDGF (lanes 1, 2, 9, and 10), the wild-type short form of SDGF (lanes 3, 4, 11, and 12), the mutant long form of SDGF defective in the signal peptide (lanes 5, 6, 13, and 14), and the mutant short form of SDGF defective in the signal peptide (lanes 7, 8, 15, and 16) were immunoblotted with monoclonal antibody 12CA5 (lanes 1–8) and this antibody adsorbed with its epitope (YPYDVPDYA) (lanes 9–16). (*c*) Cytoplasmic (lanes 1 and 3) and nuclear (lanes 2 and 4) fractions from COS cells transiently transfected with the M1 nuclear targeting mutant (*f*) long form of SDGF (lanes 1 and 2) and M1 mutant (*f*) short form of SDGF (lanes 3 and 4). (*d*) Cytoplasmic (lanes 1 and 3) and nuclear (lanes 2 and 4) fractions from COS cells transfected with the M2 nuclear targeting mutant (*f*) long form of SDGF (lanes 1 and 2) and short form of SDGF (lanes 3 and 4). (*e*) Cytoplasmic (lane 1) and nuclear (lane 2) fractions from COS cells transfected with M1/M2 (*f*) double mutant long form of SDGF. (*f*) The SDGF nuclear targeting sequence is given in single-letter code (amino acids 115–134). Mutants are named on the left; wt, wild type. (*g*) Comparison of SDGF nuclear targeting signal with other known nuclear targeting signals of *Xenopus* nucleoplasmin (NP), NO38, glucocorticoid receptor (GR), and p53 (21).

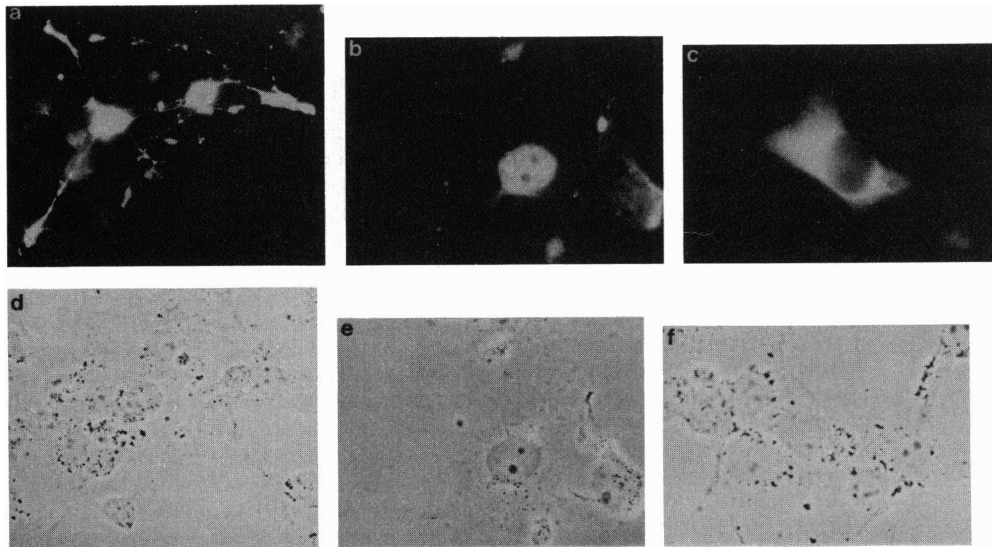

FIG. 2. Indirect immunofluorescence staining of the wild-type and the mutant SDGF gene product. The wild type and mutants of the SDGF gene were transiently expressed in COS cells and the protein was detected by indirect immunofluorescence. COS cells were transfected with wild-type SDGF (*a*), mutant SDGF defective in the secretory signal peptide (*b*), or mutant SDGF defective in the nuclear transport signal but containing the secretory signal peptide (*c*). The corresponding phase-contrast micrographs are also shown (*d*–*f*). (×370.)

(Fig. 1*d*, lanes 3 and 4), whereas the similarly mutated long form was found both in the cytoplasm and in the nucleus (Fig. 1*d*, lanes 1 and 2). Changes of the more amino-terminal basic amino acid cluster 115–119 (KRKKK) to neutral amino acids (LITLI) (M1 mutant, Fig. 1*f*) did not result in any changes in localization (Fig. 1*c*). However, the double mutation (M1/M2, Fig. 1*f*) caused even the longer form of SDGF to localize primarily in the cytoplasm (Figs. 1*e* and 2*c*). Similar results were obtained when the mutants were transfected into the human embryonic kidney cell line 293 and mouse 3T3 fibroblasts (data not shown). Therefore, these two basic amino acid clusters, which are separated by 10 amino acids, function as a nuclear targeting signal in an interdependent manner, like similar sequences in *Xenopus* nucleoplasmin, N1, NO38, glucocorticoid receptor, and p53 (Fig. 1*g*) (21).

The amino acid residues of SDGF between 173 and 194 have a potential for α-helix formation (24), and the entire array of basic residues in this region could be on the same face of an α-helix (Fig. 3*f*). This analysis, in conjunction with the finding that SDGF is actively transported into the nucleus, prompted us to study the DNA-binding properties of SDGF. The fusion proteins between glutathione *S*-transferase and

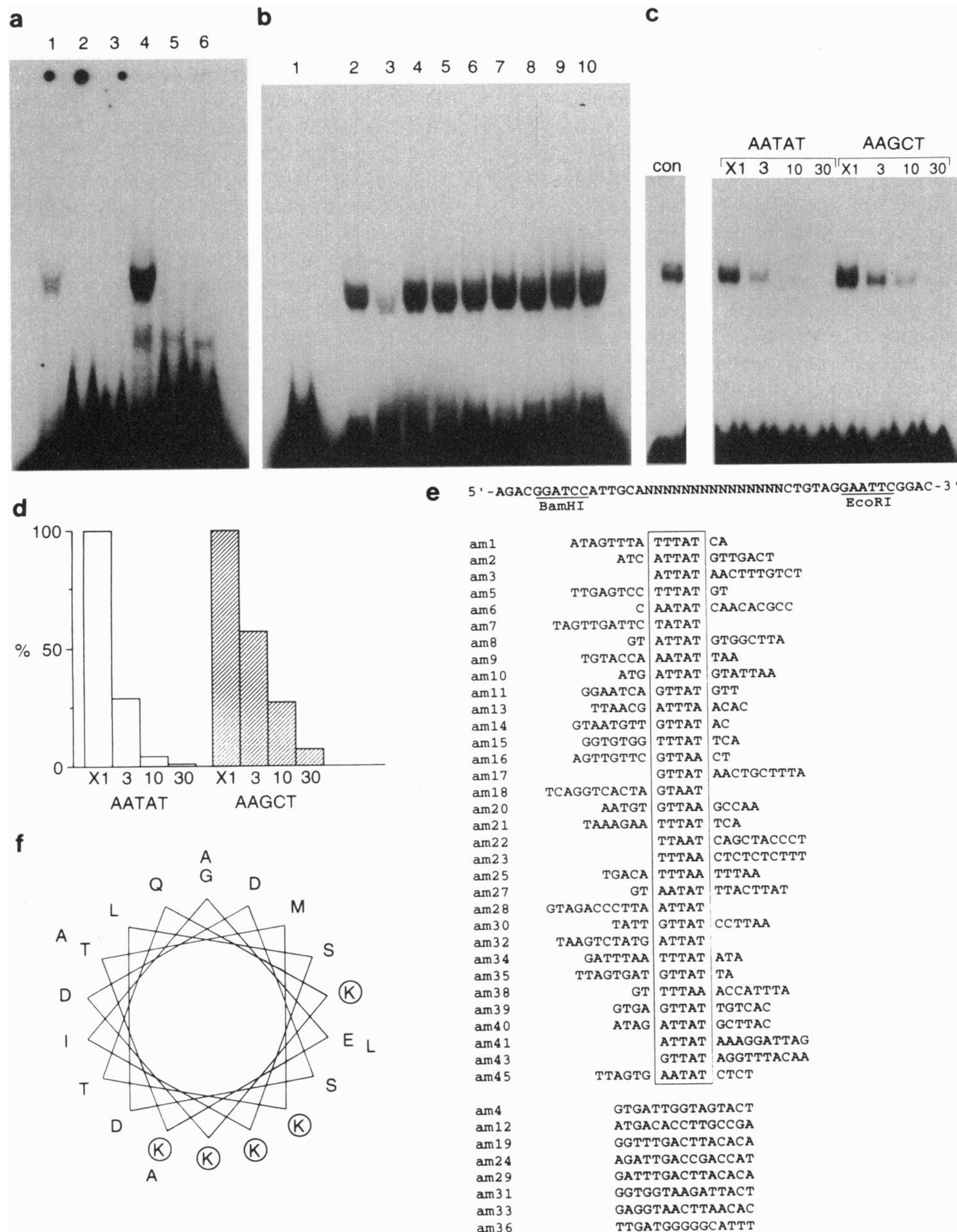

FIG. 3. Binding of glutathione *S*-transferase–SDGF fusion proteins to oligonucleotides. (*a*) Gel retardation assay of glutathione *S*-transferase with carboxyl-terminal 101 amino acids (amino acids 143–243) (lanes 1 and 4) and amino-terminal 105 amino acids (amino acids 1–105) (lanes 2 and 5) of SDGF. Fusion proteins were incubated with random oligonucleotide probes (lanes 1–3) or with probes derived from one round of selection (lanes 4–6). Lanes 3 and 6 are controls with no fusion protein. (*b*) Relative affinities of the carboxyl-terminal 101 amino acids of SDGF for the DNA binding sequences. The following probes (*e*) were used: lane 1, am1 without fusion protein; lane 2, am1; lane 3, am4; lane 4, am6; lane 5, am11; lane 6, am16; lane 7, am18; lane 8, am23; lane 9, am32; lane 10, am38. (*c*) Competition for binding of the preferred DNA sequence to the SDGF fusion protein. ^{32}P-labeled am6 oligonucleotide was incubated with SDGF fusion protein in the absence (control, con) or presence of a 1-, 3-, 10-, or 30-fold molar excess of unlabeled AATAT or AAGCT competitor oligonucleotide. (*d*) Quantification of the competition by scanning laser densitometry. Binding is expressed as the percentage of bound ^{32}P-labeled am6, relative to the amount bound in the absence of any oligonucleotide competitor (defined as 100%). (*e*) DNA sequence of oligonucleotides isolated after three rounds of binding-site selection. (*f*) Helical wheel depiction of SDGF residues 173–194 with basic residues circled.

the amino-terminal 105 amino acids (amino acids 1–105) or the carboxyl-terminal 101 amino acids (amino acids 143–243) of SDGF were constructed. A gel retardation assay was performed between the fusion proteins and a totally degenerate 15-mer oligonucleotide flanked by restriction sites (Fig. 3*e*). A weakly detectable band was obtained only in the reaction with the fusion protein containing the carboxyl-terminal 101 amino acids (Fig. 3*a*). This band was recovered and amplified by PCR. After two additional rounds of selection, the resultant oligonucleotides were cloned and sequenced. The DNA sequences of 41 independent clones were determined (Fig. 3*e*). Thirty-three out of 41 clones had the preferred sequence of (G/A/T)(T/A)TA(T/A). The remaining 8 clones did not show any obvious similarity to this sequence. When the same amounts of 9 labeled probes were applied in a gel retardation assay, all 8 clones which had the preferred sequence bound to the fusion protein strongly, whereas the clone which had no preferred sequence still bound to the fusion protein, but less well (Fig. 3*b*). To assess the sequence specificity of the DNA-binding activity of SDGF, a competitive gel retardation assay was carried out using the SDGF fusion protein and an isotopically labeled oligonucleotide containing one of the preferred sequences, AATAT. The reaction was carried out in the presence of various amounts of unlabeled AATAT or a mutant sequence AAGCT. Unlabeled AATAT competed with labeled AATAT for SDGF 3 times more effectively than the mutant sequence (Fig. 3 *c* and *d*). These data show that SDGF can bind to DNA with a relatively weak sequence specificity.

Since SDGF has nuclear targeting signals, and a mutant acidic FGF defective in a putative nuclear targeting signal is not mitogenic (25), it was asked whether or not the mitogenic activity of exogenously applied SDGF correlates with its ability to localize to the nucleus. Swiss 3T3 cells were used to investigate the relationship between the mitogenic activity and the nuclear localization of SDGF (9). HPLC-purified SDGF and the growth-conditioned medium of human 293 cells transfected with expression plasmids containing SDGF cDNA showed strong mitogenic activity on Swiss 3T3 fibroblasts, whereas the serum-free medium from cells transfected with mutant DNA defective in nuclear targeting signals had no mitogenic activity (Fig. 4*a*). Western blot analysis showed that the amount of secreted protein was the same for the wild-type and the mutant SDGF (Fig. 4*c*). Since SDGF can interact with and activate the EGF receptor (16), the abilities of the wild-type and mutant SDGFs to bind the EGF receptor were compared in a competition assay in Swiss 3T3 cells. Although less effective than EGF itself, both wild-type and mutant SDGFs competed with EGF for receptor binding at the same efficiency (Fig. 4*b*). To test the ability of the SDGF mutant defective in the nuclear targeting signal to activate the receptor, the induction of the immediate early genes c-*fos* and NGFI-A was compared between the wild-type and mutant SDGFs by Northern blot analysis (26). Both wild-type and mutant SDGFs induced c-*fos* and NGFI-A mRNAs at the same concentration as that needed for maximal induction of mitogenic activity (Fig. 4*d*). These experiments demonstrate that the mutant forms of SDGF can both bind to and activate receptor.

Tyrosine kinase activity is required to potentiate the expression of the immediate early genes (26). To study whether EGF receptor kinase activity is required for SDGF to stimulate DNA synthesis, a cell line whose EGF receptor is defective in the kinase domain was used (2). An NIH 3T3 cell line expressing the normal EGF receptor responds to the wild-type SDGF but not to the mutant SDGF defective in the nuclear targeting signals (Fig. 5*b*). The wild-type SDGF also stimulates DNA synthesis in a cell line expressing the EGF receptor kinase mutant whose initial rate of EGF internalization is similar to that of wild-type receptor (2). Although this activity is somewhat weaker than that in the cell line expressing the normal EGF receptor, it is significant (Fig. 4*c*; $P < 0.01$). Parental NIH 3T3 cells which express neither wild-type EGF receptor nor the mutant receptor respond to neither EGF nor SDGF (Fig. 5*a*). Since EGF does not have nuclear targeting signal, these data suggest that both SDGF

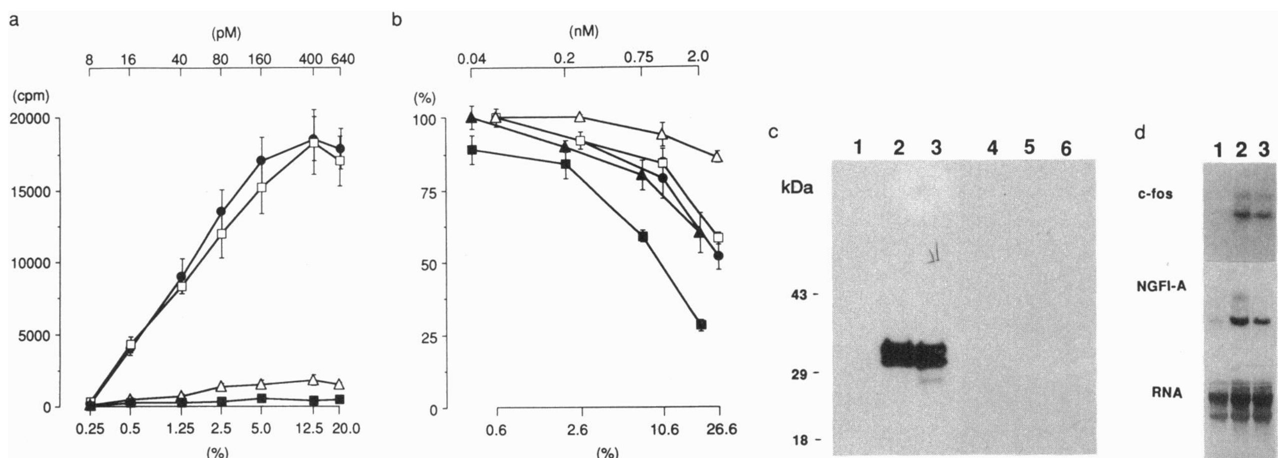

FIG. 4. Mitogenic activity and the induction of immediate early genes in Swiss 3T3 cells and binding competition of SDGF with epidermal growth factor (EGF). (*a*) Mitogenic activity of purified SDGF (●) or of growth-conditioned medium of human 293 cells transfected with the wild-type SDGF (□), the mutant SDGF defective in the nuclear targeting signals (△), or the expression plasmid pCIS2 alone (■). Activity is expressed as cpm of [^{3}H]thymidine incorporated. Concentration of purified SDGF is given in pM and that of the conditioned medium in %. Data are presented as the mean ± SEM of four determinations. The experiment was repeated five times with similar results. (*b*) Competition of ^{125}I-EGF binding with nonradioactive EGF (■), purified SDGF (▲), or growth-conditioned medium of 293 cells transfected with the wild-type SDGF (●), the mutant SDGF defective in the nuclear targeting signals (□), or the expression plasmid pCIS2 alone (△). Concentration of EGF and SDGF is given in nM and that of the conditioned medium in %. Data are presented as the mean ± SEM of four determinations. The experiment was repeated three times with similar results. (*c*) Western blot analysis of SDGF in the serum-free growth-conditioned medium from 293 cells transfected with pCIS2 expression plasmid without insert (lanes 1 and 4) or containing wild-type SDGF cDNA (lanes 2 and 5) or mutant cDNA defective in the nuclear targeting signals (lanes 3 and 6). Lanes were immunoblotted with monoclonal antibody 12CA5 (lanes 1–3) or this antibody adsorbed with its epitope (YPYDVPDYA) (lanes 4–6). (*d*) Induction of immediate early genes c-*fos* (*Top*) and NGFI-A (*Middle*) as shown by Northern blot analysis. Lanes: 1, control; 2, wild-type SDGF; 3, mutant SDGF defective in the nuclear targeting signals. RNA stained with methylene blue is also shown (*Bottom*).

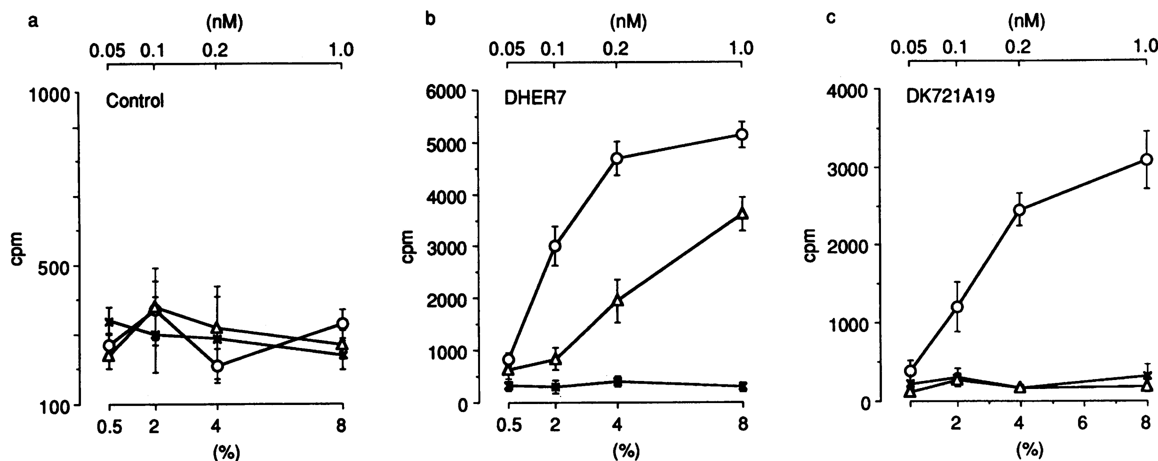

FIG. 5. Mitogenic activity of SDGF on untransfected NIH 3T3 cells (*a*) and on NIH 3T3 cell lines expressing wild-type EGF receptor (DHER7) (*b*) and mutant EGF receptor defective in the ATP-binding domain (DK721A19) (*c*). Cells were incubated with EGF (△) or with growth-conditioned medium of human 293 cells transfected with the wild-type SDGF (○) or mutant SDGF defective in the nuclear targeting signals (**X**). Each point was normalized by subtracting the activity of control 293 cell supernatant. Concentration of EGF is given in nM and that of the conditioned medium in %. Data represent the mean ± SEM of triplicate determinations.

and EGF activate the EGF receptor but that SDGF uses another signal transduction pathway to elicit its biological activity. Transport of SDGF into the nucleus is required to complete this additional signal pathway, which results in the induction of DNA synthesis.

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