MOLECULAR AND CELLULAR BIOLOGY, Jan. 1996, p. 270–280 Vol. 16, No. 1
0270-7306/96/$04.00+0
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Stimulation of Proliferation of a Human Osteosarcoma Cell Line by Exogenous Acidic Fibroblast Growth Factor Requires both Activation of Receptor Tyrosine Kinase and Growth Factor Internalization

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Received 23 March 1995/Returned for modification 3 May 1995/Accepted 19 October 1995

U2OS Dr1 cells, originating from a human osteosarcoma, are resistant to the intracellular action of diphtheria toxin but contain toxin receptors on their surfaces. These cells do not have detectable amounts of fibroblast growth factor receptors. When these cells were transfected with fibroblast growth factor receptor 4, the addition of acidic fibroblast growth factor to the medium induced tyrosine phosphorylation, DNA synthesis, and cell proliferation. A considerable fraction of the cell-associated growth factor was found in the nuclear fraction. When the growth factor was fused to the diphtheria toxin A fragment, it was still bound to the growth factor receptor and induced tyrosine phosphorylation but did not induce DNA synthesis or cell proliferation, nor was any fusion protein recovered in the nuclear fraction. On the other hand, when the fusion protein was associated with the diphtheria toxin B fragment to allow translocation to the cytosol by the toxin pathway, the fusion protein was targeted to the nucleus and stimulated both DNA synthesis and cell proliferation. In untransfected cells containing toxin receptors but not fibroblast growth factor receptors, the fusion protein was translocated to the cytosol and targeted to the nucleus, but in this case, it stimulated only DNA synthesis. These data indicate that the following two signals are required to stimulate cell proliferation in transfected U2OS Dr1 cells: the tyrosine kinase signal from the activated fibroblast growth factor receptor and translocation of the growth factor into the cell.

Acidic fibroblast growth factor (aFGF) induces a multitude of responses in target cells. In many cells, this growth factor is a potent mitogen (3, 9, 23, 24); in others, it inhibits proliferation (34) or induces various kinds of differentiation (6, 17, 27, 39, 41, 44, 50, 58). The different responses may partly be due to the fact that there are four known FGF receptor genes and a number of splicing variants for three of them (11, 22, 26, 64, 65).

In a previous paper, we showed by cell fractionation that externally added aFGF was recovered in the nuclear fraction of NIH 3T3 cells, which contain specific FGF receptors. We also presented evidence that targeting to this location is required for the stimulation of DNA synthesis, whereas external binding to the receptor is sufficient to induce tyrosine phosphorylation (62). In Vero Dr22 and U2OS Dr1 cells, which lack specific FGF receptors and do not respond to aFGF, we were able to stimulate DNA synthesis by translocating the growth factor into the cytosol as a fusion protein with the diphtheria toxin A fragment (aFGF-dtA). Reconstituted with the diphtheria toxin B fragment (dtB), the fusion protein was translocated by the diphtheria toxin pathway (42, 43) into the cytosol of Vero Dr22 and U2OS Dr1 cells, which are insensitive to the intracellular action of diphtheria toxin but rich in diphtheria toxin receptors, and it was subsequently found in the nuclear fraction (63). However, there was no stimulation of cell proliferation.

In NIH 3T3 cells, which contain FGF receptors, aFGF induced tyrosine phosphorylation, DNA synthesis, and cell proliferation; upon cell fractionation, much of the growth factor was found in the nuclear fraction, apparently because of the nuclear localization sequence contained in the N-terminal end of the growth factor (68). In fact, when this sequence was removed, there was no accumulation in the nuclear fraction and no stimulation of DNA synthesis or cell proliferation (23, 62, 68).

The fusion protein aFGF-dtA binds to the specific FGF receptors and was found to induce tyrosine phosphorylation in NIH 3T3 cells. However, in contrast to aFGF as such, it did not stimulate DNA synthesis or cell proliferation. Furthermore, the fusion protein did not accumulate in the nuclear fraction of NIH 3T3 cells (62). Our interpretation of these data was that aFGF alone is able to translocate to the nucleus and stimulate DNA synthesis, whereas the fusion protein is unable to be translocated into cells by the aFGF pathway.

To test the hypothesis that both activation of the tyrosine kinase of FGF receptors and translocation of aFGF to the cytosol and nucleus are required for cell proliferation, we transfected U2OS Dr1 cells with FGF receptor 4 (FGFR4) to obtain cells with receptors for both the growth factor and diphtheria toxin. We then tested the ability of aFGF-dtA alone and after reconstitution with dtB ([aFGF-dtA+B]) to stimulate tyrosine phosphorylation, DNA synthesis, and cell proliferation in the transfected cells. The results indicate that both tyrosine phosphorylation and translocation of the growth factor into cells are required to stimulate cell proliferation.

MATERIALS AND METHODS

Materials, buffers, and plasmids. [^{35}S]methionine (1,000 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol), and $Na_3{}^{32}PO_4$ were from Amersham. The HEPES medium used was bicarbonate-free Eagle's minimum essential medium buffered with 20 mM HEPES (*N*-2-hydroxethylpiperazine-*N*′-2-ethanesulfonic acid) to pH 7.4. The phosphate-buffered saline (PBS) used was 140 mM NaCl–10 mM

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Na_2HPO_4 (pH 7.2). The lysis buffer used was PBS (pH 7.2) containing 10 mM EDTA, 1% Triton X-100, 10 mM NaF, 0.1 mM sodium vanadate, 200 U of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-ethylmaleimide. P lysis buffer contained 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium PP_i, 100 μM sodium orthovanadate, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The monoclonal anti-human FGFR1 antibody (flg) was from Upstate Biotechnology, Lake Placid, N.Y.; the rabbit anti-human FGFR2 antibody (bek) was from Santa Cruz Biotechnology; and basic FGF was from R&D Systems, Minneapolis, Minn.

The expression plasmid LTRFGFR4 encoding FGFR4 and anti-FGFR4 (46) were kind gifts from K. Alitalo. Plasmid LTRFGFR4 (46) was cut with *Sma*I and religated to give pFGFR4-Δsma. This plasmid encodes an FGFR4 molecule from which most of the cytoplasmic domain, including the whole kinase domain, is deleted. It ends with amino acid Arg-464, followed by the sequence Val-Thr-Leu-Ser-Leu-Ala-Gly-Glu-Leu-Leu-Cys. pMamNeo, which encodes the neomycin phosphotransferase gene, was obtained from Clontech. pBD-30 (encoding dtA) and pBD-23 (encoding dtB) (56), pHBGF-1α (encoding aFGF) (23), pH BFG-dt1 (encoding aFGF in front of dtA) (63), and pHBGF-dt10 [encoding aFGF in front of dtA with an internal disulfide (dtA-SS) (15)] have been described elsewhere (62).

pTrc-aFGF-dtA was obtained by cloning the fusion protein aFGF-dtA (63) into the *Nco*I and *Eco*RI sites of pTrc99A (Pharmacia, Uppsala, Sweden). To obtain pTrc-aFGF-dt, a fragment obtained from pBD-1 (33) (encoding the essentially nontoxic Glu-148–Ser mutant [because the full-length, active diphtheria toxin is considered hazardous to clone]) by cutting with *Eco*RI, filling in with T4 polymerase, and then cutting with *Cel*II was cloned into pTrc-aFGF-dtA cut with *Sal*I, and the *Sal*I site was filled in with T4 polymerase and then cut with *Cel*II. HBGF-1α in pET-3c (23) was a kind gift from T. Maciag.

Purification of recombinant proteins. Bacterial pellets were sonicated and centrifuged. The clear supernatant was applied to a heparin cartridge (Bio-Rad) equilibrated with 0.5 M NaCl in 20 mM sodium phosphate (pH 7.5)–1 mM EDTA–1 mM dithiothreitol. Fusion proteins were eluted with 1 M NaCl in the same buffer and dialyzed against 20 mM sodium phosphate (pH 8.0)–1 mM EDTA–1 mM dithiothreitol. Subsequently, the fusion proteins were applied to a Q cartridge (Bio-Rad) and eluted with a linear NaCl gradient in the same buffer.

In vitro transcription and translation. Plasmids linearized downstream of the inserts by *Eco*RI were transcribed in vitro with T3 polymerase (33, 62). The transcripts were translated for 1 h at 30°C in a rabbit reticulocyte lysate system (Promega, Madison, Wis.) in the presence of 1 μM [^{35}S]methionine (1,000 Ci/mmol; Amersham). For the preparation of unlabelled proteins, instead of [^{35}S]methionine, 25 μM unlabelled methionine was used. The concentrations of translation products obtained were estimated as previously described (56). After translation, the lysates were dialyzed at 4°C first for 16 h against PBS and then for 4 h against HEPES medium to remove free [^{35}S]methionine and the reducing agent, allowing disulfide bridges to be formed. The translation mixtures used were either undiluted or diluted with HEPES medium (pH 7.2).

Cell culture. Cells were maintained and propagated under standard conditions (5% CO_2 in Eagle's minimal essential medium containing 5% fetal calf serum [FCS]). Two days prior to the experiment, cells were seeded on 12- and 24-well Costar plates at densities of 10^5 and 5×10^4 cells per well, respectively.

Transfection of cells. U2OS Dr1 cells were seeded on 10-cm-diameter petri dishes (10^6 cells per dish) in Dulbecco modified minimal essential medium (DMEM) containing 5% FCS. The next day, cells were washed in serum-free medium, and then 2.5 ml of serum-free DMEM containing 5 μg of LTRFGFR4 and 5 μg of pMamNeo DNA and 20 μl of a 1-mg/ml aqueous solution of DOTAP (Boehringer Mannheim) previously vortexed and left for 5 min at room temperature were added. Cells were incubated for 5 h at 37°C with occasional careful shaking. Then 250 μl of FCS and 2.5 ml of medium containing 5% FCS were added, and cells were incubated overnight. The next day, the medium was changed and cells were trypsinized, diluted 1:3, and seeded on new petri dishes. After 4 days, the medium was removed and DMEM containing 5% FCS and 1 mg of geneticin per ml was added. Small colonies developed after 2 weeks. Cells were then incubated further in DMEM containing 0.5% FCS and 10 ng of aFGF per ml. Colonies that grew under these conditions were tested for aFGF-stimulated incorporation of [^{3}H]thymidine, as previously described (62). One transfectant (U2OS Dr1 R4) was selected.

For transfection with pFGFR4-Δsma, the same transfection method was used, except that the appearing colonies were screened for the binding of aFGF, as well as reverse transcription-PCR with primers specific for the extracellular domain of the receptor.

Analysis of cellular mRNA. Total RNA was isolated from cells, converted into DNA with reverse transcriptase, and amplified by PCR by the method of Brogi et al. (7). For Northern (RNA) blot analysis of c-*fos* induction, NIH 3T3, U2OS Dr1, and U2OS Dr1 R4 cells were seeded in 25-cm^2 tissue culture flasks at a density of 10^6 cells per flask in DMEM containing 10% FCS. On the following day, the medium was changed to DMEM without FCS, and cells were grown for 48 h. Cells were subsequently incubated for 30 min at 37°C in HEPES medium with various additions, and total RNA was isolated from cells by the LiCl-urea-sodium dodecyl sulfate (SDS) method (4). Total RNA samples (5 μg) were separated on 1% agarose–formaldehyde gels (49) and blotted onto Hybond-N membranes (Amersham) according to the manufacturer's instructions. The membranes were baked at 80°C for 2 h and UV cross-linked. The blots were hybridized (12) with a v-*fos* probe (1.1-kb *Pst*I-*Bgl*II fragment) (14) labelled with ^{32}P by the random primer technique (16). Filters were exposed to Kodak (Rochester, N.Y.) X-Omat AR film at −80°C in the presence of an intensifying screen.

Assay for tyrosine phosphorylation. Cells near confluence that had been incubated for 24 h without serum were incubated for 3 h at 37°C with 0.25 mCi of $Na_3{}^{32}PO_4$ per ml in 25 mM HEPES (pH 7.4) containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, and 0.1% bovine serum albumin (BSA). Then cells were treated with constructs translated in vitro or with purified recombinant constructs for 10 min at 37°C. After treatment, cells were washed once with ice-cold PBS (pH 7.4) containing 50 mM NaF, 30 mM sodium PP_i, and 100 μM sodium orthovanadate and lysed in P lysis buffer. Lysates were centrifuged twice in an Eppendorf centrifuge at 14,000 rpm for 10 min at 4°C. Supernatants were rotated for 2 h at 4°C with 25-μl aliquots of monoclonal anti-phosphotyrosine antibody (Boehringer Mannheim) coupled to Sepharose 4B. The Sepharose beads were washed four times with P lysis buffer, and then phosphotyrosine-containing proteins were specifically eluted from the beads with 10 mM phenylphosphate in 10 mM Tris-HCl (pH 7.4)–50 mM NaCl–0.1% Triton X-100–0.1% BSA. The eluted material was diluted with 2 volumes of 10 mM Tris-HCl (pH 7.4)–0.1% Triton X-100–0.1% BSA and rotated for 1 h at 4°C with 25-μl aliquots of rabbit FGFR4 antibody (provided by K. Alitalo) coupled to protein A-Sepharose (Pharmacia). The Sepharose pellets were subsequently washed three times with the same buffer and once with water, extracted with sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

In mitogen-activated protein (MAP) kinase activation assays, starved, nearly confluent cells were treated with aFGF and aFGF-dtA as described above. Postnuclear fractions were rotated for 1 h at 4°C with 15-μl aliquots of monoclonal anti-phosphotyrosine agarose (Sigma, St. Louis, Mo.). Materials specifically eluted from agarose with 10 mM phenylphosphate were subjected to immunoprecipitation with monoclonal anti-MAP kinase (ERK 1+2) antibody (Zymed) coupled to Sepharose 4B. The Sepharose pellets were washed and extracted with sample buffer and analyzed by SDS-PAGE.

SDS-PAGE. SDS-PAGE was carried out by the method of Laemmli (31). Gels were fixed in 4% acetic acid–27% methanol for 30 min and, in the case of proteins labelled with [^{35}S]methionine, treated with 1 M sodium salicylate (pH 5.8) in 2% glycerol for 30 min. Dried gels were exposed to Kodak XAR-5 film in the absence of intensifying screens at −80°C for autoradiography or fluorography.

Immunoblots. Cells near confluence that had been starved of serum for 24 h were treated with different constructs for 10 min at 37°C. Cells were washed in ice-cold PBS containing 0.1 mM sodium orthovanadate, lysed in supernatant SDS sample buffer, and subjected to reducing SDS-PAGE on 8% minigels. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell). After transfer, membranes were blocked with 1% casein–2% BSA in Tris-buffered saline and then incubated with anti-phosphotyrosine (Sigma), monoclonal anti-human FGFR1 (Upstate Biotechnology), or rabbit anti-FGFR4. Next, blots were incubated with a second antibody labelled with alkaline phosphatase (Promega) and visualized by incubation with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium)-nitroblue tetrazolium according to the manufacturer's instructions.

Binding and uptake of [^{35}S]methionine-labelled aFGF. To measure binding, dialyzed translation mixture (diluted five times in HEPES medium) was added to cells growing as monolayers on 12-well microtiter plates and kept at 24°C for 20 min in the presence of 1 mM unlabelled methionine (33). To measure uptake, cells were incubated with 10 μl of labelled translation mixture in 1 ml of DMEM for 24 h. In some cases, cells were then washed and incubated for an additional 24 h. Finally, cells were analyzed by SDS-PAGE and fluorography, as previously described (37).

Binding of ^{125}I-labelled aFGF and the ability of fusion proteins to compete for binding. Confluent cells growing on 12-well gelatinized microtiter plates were washed twice with ice-cold binding buffer (DMEM containing 50 mM HEPES [pH 7.4], 0.2% gelatin, and 10 U of heparin per ml). Cells were incubated with ^{125}I-aFGF for 4 h at 4°C. Then they were washed twice with binding buffer, twice with PBS, and twice with 1 M NaCl. Cells were lysed in 0.5 M NaOH, and the solubilized radioactivity was measured.

Nonspecific binding was estimated by the incubation of cells in the presence of a 100-fold molar excess of unlabelled pure recombinant aFGF. Receptor dissociation constants were estimated by the method of Scatchard (51). Recombinant aFGF was iodinated by the Iodogen method (18), and labelled aFGF was purified on a heparin-Sepharose column. The specific activity of ^{125}I-aFGF was 15,000 cpm/ng.

Fractionation of cells and immunoprecipitation with anti-aFGF. As indicated, cells were fractionated into cytoplasmic and nuclear fractions and immunoprecipitated before SDS-PAGE as follows: cells were lysed in lysis buffer and centrifuged twice for 5 min at 14,000 rpm and 4°C in an Eppendorf centrifuge. The supernatant (cytoplasmic fraction) was rotated for 2 h at 4°C with 30 μl of protein A-Sepharose CL-4B previously treated with 2 μl of rabbit anti-bovine aFGF (Sigma). After being washed, the adsorbed material was analyzed by SDS-PAGE. The nuclear pellets were washed twice by resuspension in lysis buffer containing 0.3 M sucrose and 1 mM $MgCl_2$, layered over 0.8 ml of 0.7 M sucrose, and centrifuged for 15 min at 3,000 rpm and 4°C in an Eppendorf centrifuge. Then nuclei were extracted by treatment with 0.5 M NaCl in PBS and

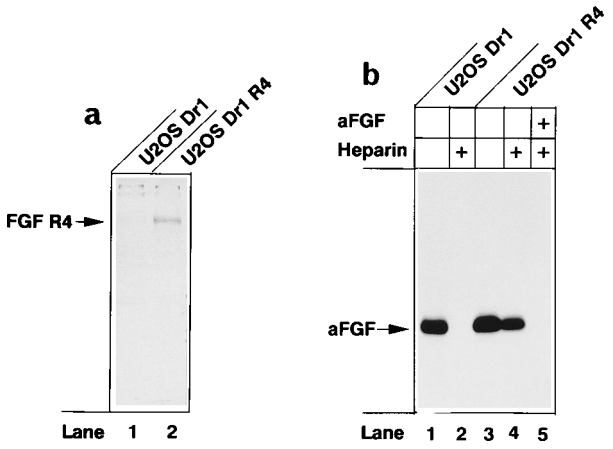

FIG. 1. Expression of FGFR4 in U2OS Dr1 cells induces specific aFGF binding. (a) Untransfected (U2OS Dr1) and transfected (U2OS Dr1 R4) cells were lysed, and the postnuclear supernatant was subjected to reducing SDS-PAGE. The proteins were transferred to nitrocellulose membranes, which were then probed with antiserum against the C-terminal 16 amino acids of FGFR4. The bound antibodies were visualized with anti-rabbit immunoglobulin linked to alkaline phosphatase. (b) Untransfected and transfected cells were incubated with 10 ng of [^{35}S]methionine-labelled aFGF per ml for 20 min at 24°C. In some cases, 50 U of heparin per ml and 15 μg of unlabelled aFGF per ml were present (+). Cells were then washed and analyzed by reducing SDS-PAGE and fluorography.

sonication. Finally, the sonicated material was diluted five times in PBS containing 0.1% Triton X-100 and subjected to immunoprecipitation as described above.

Measurements of DNA synthesis. Cells growing on 24-well microtiter plates (5×10^4 or 10^5 cells per well) were preincubated for 48 h in serum-free medium at 37°C. Cells were treated with increasing amounts of aFGF, fusion protein, or FCS, and incubation was continued for 24 h at 37°C. During the last 6 h, cells were incubated with 1 μCi of [^{3}H]thymidine per ml, as previously described (23), and the incorporated radioactivity was measured. In one case, cells were starved of serum by incubation for 72 h in medium containing 0.5% FCS and treated with [aFGF-dtA+B] from 12 to 72 h in the same medium, and then [^{3}H]thymidine incorporation was measured, as described above.

RESULTS

U2OS Dr1 cells transfected with FGFR4 bind aFGF specifically. U2OS Dr1 cells are rich in receptors for diphtheria toxin, but they are resistant to the toxic effect because of the inability of the elongation factor 2 of these cells to be ADP ribosylated by dtA (57, 62). These cells lack functional receptors for aFGF (62). U2OS Dr1 cells were transfected with two plasmids, one carrying cDNA for FGFR4 (46, 60) and the other one carrying a gene providing neomycin resistance. Geneticin-resistant clones that grew after 2 weeks were tested for the ability to grow in low-serum medium supplemented with aFGF. One transfected clone (U2OS Dr1 R4) was selected.

Western blot (immunoblot) analysis of cells was carried out with antibodies against the C-terminal 16 amino acids of FGFR4 (46). The results (Fig. 1a, lane 2) show a band in U2OS Dr1 R4 with an apparent molecular mass of 110 kDa, as expected for FGFR4 (46). This band was absent from untransfected cells (Fig. 1a, lane 1).

[^{35}S]methionine-labelled aFGF (62) was added to transfected cells to test for specific binding. As shown in Fig. 1b, the labelled growth factor bound both to untransfected (lane 1) and transfected (lane 3) cells. However, while heparin prevented the binding to untransfected cells (Fig. 1b, lane 2), it merely reduced it for transfected cells (lane 4). The combination of heparin and excess unlabelled aFGF prevented this binding (Fig. 1b, lane 5). These data indicate that in untransfected cells, the growth factor binds to surface heparans, whereas in transfected cells, it also binds to specific FGF receptors.

Analysis of cellular RNA by reverse transcription-PCR, as described by Brogi et al. (7), showed the presence of mRNA for FGFR4 in transfected cells, not in untransfected cells (data not shown). In addition, mRNAs for FGFR1 and FGFR2 were detected both in untransfected and transfected U2OS Dr1 cells. Since we were unable to demonstrate specific aFGF binding in untransfected cells, this mRNA cannot be expressed as protein to any significant extent. In none of the cells were we able to demonstrate FGFR1 and FGFR2 on Western blots with anti-flg (48) and anti-bek antibodies (data not shown).

We also detected small amounts of mRNA for aFGF and its shorter splicing variant (67) in U2OS Dr1 and U2OS Dr1 R4 cells, not in NIH 3T3 cells. In none of these cell lines could we detect endogenous aFGF on Western blots in which the heparin binding material from 2×10^6 cells was probed with anti-aFGF. Nor could we demonstrate any immunoprecipitable labelled aFGF in lysed [^{35}S]methionine-labelled cells or the concentrated medium from 24 ml of cell culture (data not shown).

Abilities of aFGF and the fusion protein aFGF-dtA to bind to and induce tyrosine phosphorylation in transfected cells. In the following experiments, we used aFGF and fusion proteins of aFGF and either dtA or dtA-SS. In the latter fusion construct, a disulfide bridge has been introduced into dtA, rendering dtA translocation incompetent by the diphtheria toxin pathway (15). Reconstituted with dtB, aFGF-dtA, not aFGF-dtA-SS, could be translocated into cells containing diphtheria toxin receptors (62, 63). The recombinant proteins were expressed either in a reticulocyte lysate system or in bacteria. In the latter case, the proteins were purified to homogeneity before being used.

Binding experiments showed that ^{125}I-aFGF was bound in a saturable way to U2OS Dr1 R4 cells, with a K_d of 152 ± 22 pM and 46,000 binding sites per cell, whereas there was no measurable specific binding to the parent U2OS Dr1 cells (Fig. 2a). To test the abilities of these constructs to bind to cells, competition binding with ^{125}I-aFGF was carried out. aFGF-dtA was about 5-fold less efficient than aFGF alone in competing for the binding of ^{125}I-aFGF to U2OS Dr1 R4 cells, whereas aFGF fused to full-length diphtheria toxin (aFGF-dt) was about 10-fold less efficient. Similar results were obtained with NIH 3T3 cells (Fig. 2c). For comparison, we also tested basic FGF, which was a less efficient competitor (Fig. 2b), in accordance with earlier reports that it binds to FGFR4 with 10- to 100-fold-lower affinity than does aFGF (47, 62). In the case of NIH 3T3 cells, it was also less efficient than aFGF was (Fig. 2c).

We have previously shown that while aFGF induced extensive tyrosine phosphorylation in NIH 3T3 cells, it did not induce measurable tyrosine phosphorylation in Vero Dr22 or U2OS Dr1 cells, which lack functional receptors for aFGF (62). Western blots with anti-phosphotyrosine showed that a band corresponding to ~110 kDa appeared in transfected U2OS Dr1 R4 cells treated with aFGF, [aFGF-dtA+B], and [aFGF-dtA-SS+B], but it was not detected in untreated cells (data not shown).

We also carried out experiments in which cells were labelled with $Na_3{}^{32}PO_4$ and the detergent-soluble fraction was treated with immobilized anti-phosphotyrosine. The specifically adsorbed material was eluted with phenylphosphate and subsequently adsorbed onto immobilized antibodies against the C-terminal 16 amino acids of FGFR4. As shown in Fig. 3a, no labelled material was immunoprecipitated in untransfected cells treated with aFGF and the various fusion proteins (lanes

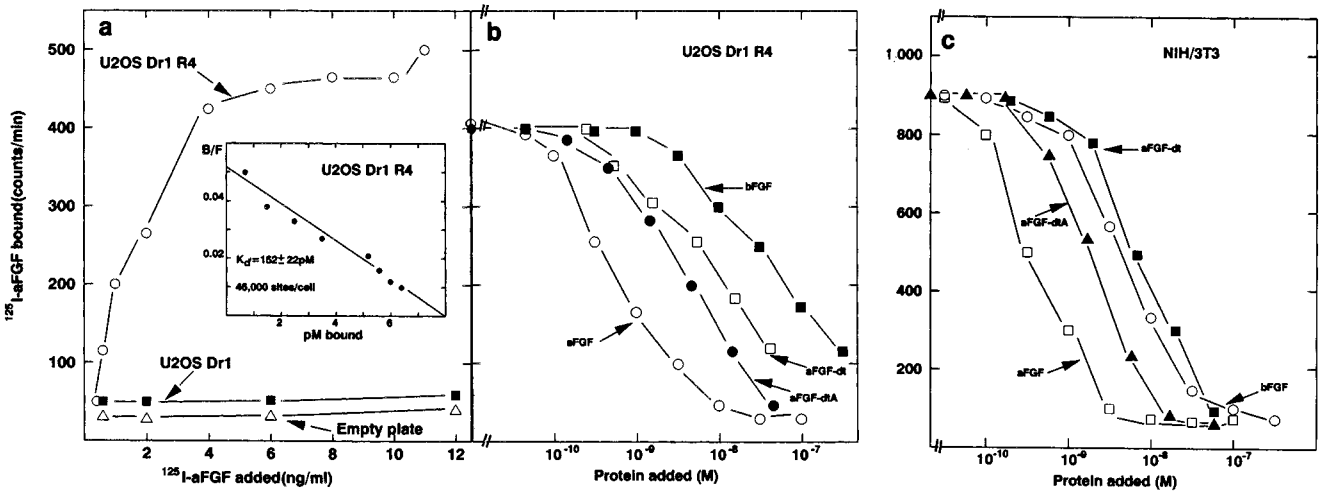

FIG. 2. Abilities of different constructs to bind to U2OS Dr1 R4 cells. (a) Increasing amounts of ^{125}I-aFGF were added to U2OS Dr1 and U2OS Dr1 R4 cells growing on 24-well microtiter plates coated with gelatin. After 4 h at 4°C, cells were washed and dissolved in 0.5 M NaOH, and the cell-associated radioactivity was measured. The inset is a Scatchard plot of the binding data for U2OS Dr1 R4 cells. (b and c) Abilities of aFGF, aFGF-dtA, aFGF-dt, and basic FGF (bFGF) to compete with ^{125}I-aFGF for binding to U2OS Dr1 R4 and NIH 3T3 cells. ^{125}I-aFGF (6 ng/ml) was added to U2OS Dr1 R4 or NIH 3T3 cells in the presence of increasing concentrations of competing proteins, as indicated, and the amount of radioactivity bound was measured as described for panel a.

1 to 6), whereas a labelled ~110-kDa protein, which presumably represents the receptor (lanes 8 to 13), was immunoprecipitated from transfected (U2OS Dr1 R4) cells. In addition, 10% FCS induced the labelling of this protein in transfected cells. In the absence of additions or in the presence of reticulocyte lysate or diphtheria toxin alone, there was no labelling of this band (lanes 15 to 17). At a low concentration (0.1 ng/ml), aFGF-dtA was less efficient than was aFGF alone in inducing the labelling of the ~110-kDa protein (Fig. 3b), in accordance with the somewhat lower affinity (Fig. 2). However, at higher concentrations (1 to 10 ng/ml) there was little difference.

In other experiments, we immunoprecipitated FGF receptors and carried out immunoblots with anti-phosphotyrosine. In these experiments, aFGF and aFGF-dtA were also approximately equally efficient in inducing phosphorylation of the receptor (data not shown).

Both aFGF and aFGF-dtA were able to induce tyrosine phosphorylation of MAP kinase in transfected cells, not in untransfected cells (Fig. 3c). Similar results were obtained with pure recombinant aFGF-dt (data not shown). At a concentration of 10 ng/ml, neither aFGF nor [aFGF-dtA+B] induced measurable amounts of c-*fos* in U2OS Dr1 R4 cells, while there was a strong induction by both in NIH 3T3 cells, which contain FGFR1 and FGFR2 (Fig. 3d). In NIH 3T3 cells at 1 ng/ml, these two proteins induced equally strong labelling (data not shown). These findings are in accordance with observations made for other cells transfected with FGFR4 by other authors, who found the induction of MAP kinase (although less than that in cells transfected with FGFR1) and no measurable induction of c-*fos* (60, 61).

Taken together, the results indicate that little or no FGF receptor is expressed as protein in untransfected U2OS Dr1 cells and that FGFR4 is expressed in transfected cells and is able to be autophosphorylated upon the treatment of cells with aFGF and its fusion proteins.

Translocation of aFGF and aFGF-dtA to the nuclear fraction. To test if labelled aFGF was transferred to the nuclear fraction in transfected cells, U2OS Dr1 and U2OS Dr1 R4 cells were incubated with [^{35}S]methionine-labelled aFGF, lysed, and then separated into cytoplasmic and nuclear fractions. As shown in Fig. 4, after a 24-h incubation with [^{35}S]methionine-labelled aFGF most of the labelled material was found in the cytoplasmic fraction of untransfected cells (lane 1), probably representing material bound to the surface heparans or within endocytic vesicles. In transfected cells, a considerable amount was also present in the nuclear fraction (Fig. 4, lane 6). When cells were washed and further incubated for 24 h in medium without labelled aFGF, very little material was associated with untransfected cells (Fig. 4, lanes 3 and 4), whereas a considerable amount was found in transfected cells (lanes 7 and 8). In this case, most of the material was found in the nuclear fraction. This suggests that in transfected cells, aFGF is able to enter the nucleus, as previously found for NIH 3T3 cells (23, 62), whereas this is not the case in cells lacking specific FGF receptors.

The fusion protein aFGF-dtA was previously found to be unable to enter the nuclear fraction of NIH 3T3 cells, in spite of the fact that it was specifically bound to the FGF receptors (62). The data in Fig. 5 show that it also did not enter the nuclear fraction of U2OS Dr1 cells or their transfected counterparts. After 24 h of incubation with the fusion protein, labelled aFGF-dtA was associated with both cell types (Fig. 5, lanes 1 and 3) and apparently bound to surface heparans in U2OS Dr1 cells and to surface heparans as well as to FGFR4 in U2OS Dr1 R4 cells. However, when cells were washed and further incubated for 24 h in the absence of aFGF-dtA, no labelled material remained in any cell line (Fig. 5, lanes 2 and 4).

When the fusion protein was reconstituted with dtB to yield [aFGF-dtA+B] and to allow the entry of aFGF-dtA by the diphtheria toxin pathway, the fusion protein remained in cells after 48 h (Fig. 5, lanes 5 and 7). On the other hand, when heparin was present to prevent the unfolding of aFGF in the fusion protein and therefore translocation (62, 63), there was no retention of labelled material in cells (Fig. 5, lanes 6 and 8). Uptake occurred in both untransfected and FGFR4-transfected cells, as it depended only on the presence of diphtheria toxin receptors.

We have previously shown that when a disulfide bridge is introduced into dtA (dtA-SS) to inhibit the unfolding of dtA,

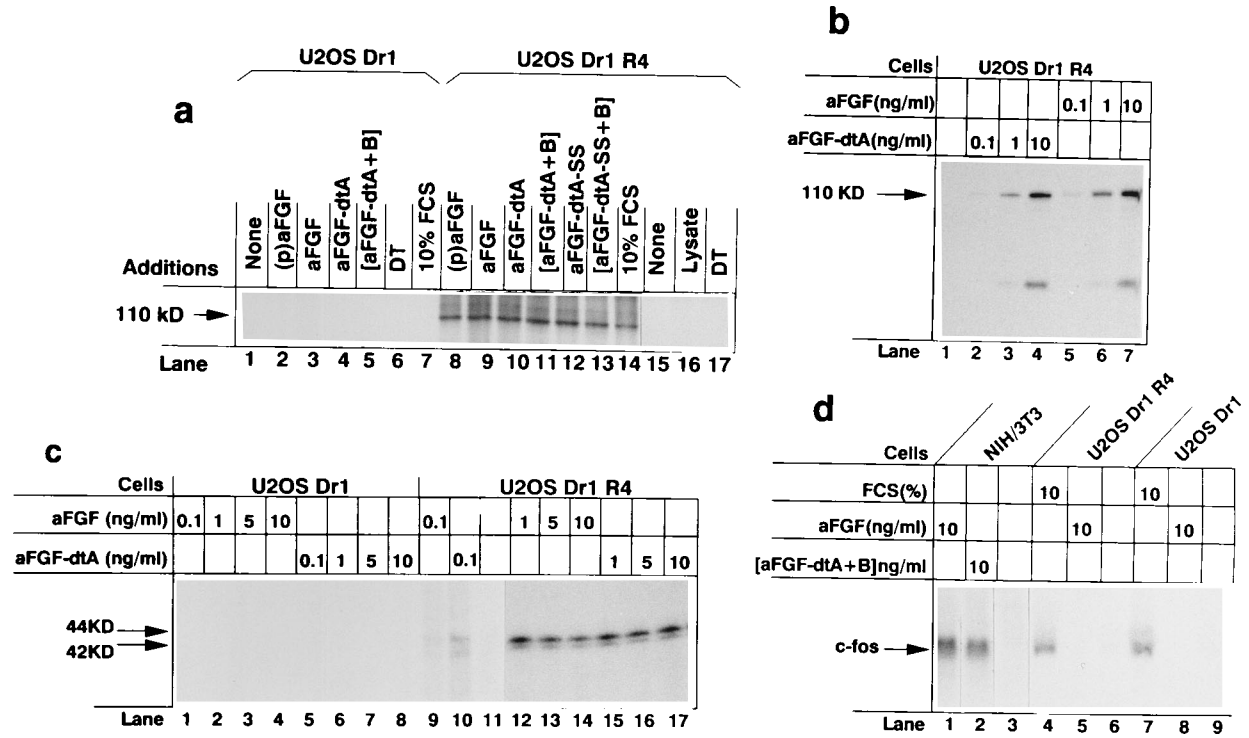

FIG. 3. Abilities of different constructs to induce tyrosine phosphorylation and c-*fos* expression in different cells. (a) Serum-starved U2OS Dr1 and U2OS Dr1 R4 cells, as indicated, were preincubated with $Na_3{}^{32}PO_4$ for 3 h and then with 5 ng of the indicated additions per ml for 10 min. Subsequently, cells were lysed and the postnuclear supernatant was treated with Sepharose-bound anti-phosphotyrosine. The adsorbed material was eluted with phenylphosphate, adsorbed to Sepharose-bound anti-FGFR4, and analyzed by reducing SDS–7.5% PAGE and autoradiography. None, no addition; aFGF and aFGF-dtA, the corresponding constructs translated in a rabbit reticulocyte lysate system; (p)aFGF, purified recombinant aFGF; DT, diphtheria toxin reconstituted by dialyzing together dtA and dtB synthesized in the reticulocyte lysate system; Lysate, 10 μl of reticulocyte lysate alone (which represents the highest amount of lysate added with in vitro-synthesized proteins). (b) Increasing amounts of aFGF and aFGF-dtA were added to U2OS Dr1 R4 cells, and tyrosine phosphorylation of FGFR4 was measured as described for panel a. (c) Increasing amounts of aFGF, aFGF-dtA, or aFGF-dt were added to U2OS Dr1 or U2OS Dr1 R4 cells, as indicated, and cells were processed as described for panel a, except that anti-MAP kinase was used. (d) Serum-starved cells were incubated for 30 min at 37°C in HEPES medium with the indicated additions. To control samples was added an amount of reticulocyte lysate without recombinant protein that was equal to the amount of lysate added with the highest concentrations of aFGF and aFGF-dtA. Total RNA was isolated from cells, and after electrophoresis and Northern transfer, c-*fos* expression was detected by hybridization to a ^{32}P-labelled v-*fos* probe.

translocation to the cytosol is prevented (15). There was also no translocation of the fusion protein when [aFGF-dtA-SS+B] was given to transfected cells (62). The data in lanes 9 and 10 of Fig. 5 show that in neither untransfected nor transfected cells did this construct remain in cells after 48 h.

In attempts to determine where the labelled material associated with cells after 48 h was located, cells were fractionated into cytoplasmic and nuclear fractions. The data in lanes 11 to 14 of Fig. 5 show that in both cell lines, most of the fusion protein was present in the nuclear fraction, in accordance with our previous findings with untransfected cells (62). The finding that there was less translocated material in transfected cells (Fig. 5, lanes 7 and 14) than in untransfected cells (lanes 5 and 12) is probably due to interactions of parts of the fusion proteins with both receptors (dtB with the diphtheria toxin receptor and aFGF-dtA with FGFR4), which may prevent translocation.

Taken together, these data indicate that the fusion protein aFGF-dtA is able to enter cells only when reconstituted with dtB to yield [aFGF-dtA+B] and that once inside the cell it is translocated to the nucleus where it is quite stable.

Abilities of different constructs to stimulate DNA synthesis and cell proliferation. To test whether aFGF and its fusion proteins stimulate DNA synthesis, untransfected and transfected cells were serum starved and then incubated with the different proteins for 24 or 48 h, as indicated. Finally, the

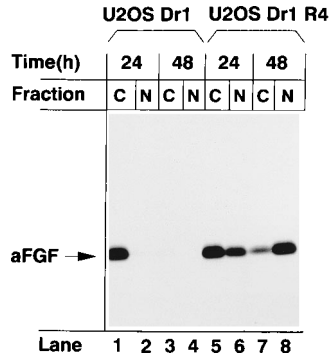

FIG. 4. Uptake of aFGF in the nuclear fraction of untransfected U2OS Dr1 cells and cells transfected with FGFR4. Reticulocyte lysate containing [^{35}S]methionine-labelled aFGF (10 μl/ml of medium) was added to cells growing as monolayers on 12-well microtiter plates. Cells were incubated with reticulocyte lysate for 24 h at 37°C, washed, and then either harvested (lanes 1, 2, 5, and 6) or incubated in medium without growth factor for an additional 24 h at 37°C before being harvested (lanes 3, 4, 7, and 8). Cells were harvested by scraping, lysed, fractionated, and immunoprecipitated with anti-aFGF antibody, as described in Materials and Methods. The precipitated proteins from the cytoplasmic fraction (C) and sonicated nuclear fraction (N) were analyzed by reducing SDS-PAGE (13.5% polyacrylamide) and fluorography. Each lane contained materials from two wells (i.e., 10^6 cells).

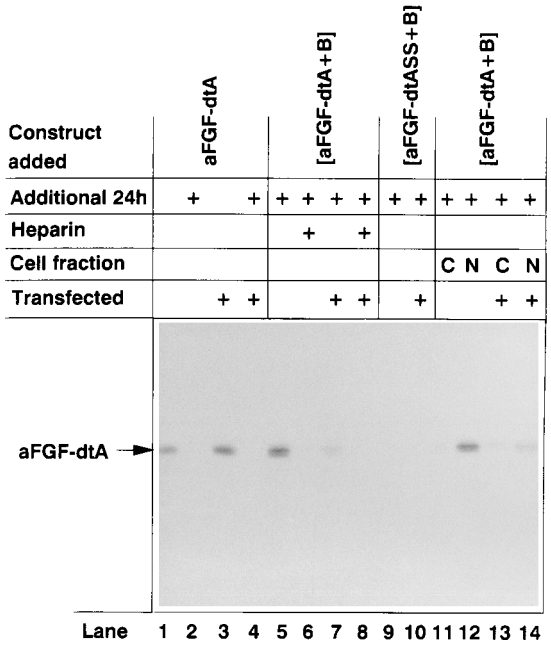

FIG. 5. Translocation of fusion protein into cells and the nuclei of untransfected and transfected U2OS Dr1 cells. [^{35}S]methionine-labelled constructs were added to cells, as indicated, incubated at 37°C for 24 h, washed three times, and then either harvested (lanes 1 and 3) or incubated in medium without these constructs for an additional 24 h at 37°C before being harvested (lanes 2 and 4 to 14). In some cases, 15 U of heparin per ml was present throughout the incubation (lanes 6 and 8). Cells were then washed and lysed, and the total trichloroacetic acid-precipitable material was collected. As indicated, the lysate was fractionated into cytoplasmic (C) and nuclear (N) fractions and immunoprecipitated, as described in the legend to Fig. 4. The material was finally analyzed by nonreducing SDS-PAGE.

abilities of cells to incorporate [^{3}H]thymidine were measured. The results in Fig. 6a show that aFGF stimulated [^{3}H]thymidine incorporation in transfected U2OS Dr1 R4 cells, not in untransfected U2OS Dr1 cells. In NIH 3T3 cells, aFGF stimulated [^{3}H]thymidine incorporation, in accordance with previous findings (23).

The addition of diphtheria toxin together with aFGF did not inhibit the stimulation of [^{3}H]thymidine incorporation, demonstrating that all of these cell lines were resistant to diphtheria toxin (Fig. 6b). U2OS Dr1 and U2OS Dr1 R4 cells were also resistant to *Pseudomonas* exotoxin A, which acts at the same intracellular target as diphtheria toxin does but binds to a different receptor (45). This is in accordance with the finding that the elongation factor 2 of these cells cannot be ADP ribosylated by these toxins (57, 62). On the other hand, the incorporation of [^{3}H]thymidine in NIH 3T3 cells was strongly inhibited, in accordance with the fact that although they lack diphtheria toxin receptors, murine cells are highly sensitive to *Pseudomonas* exotoxin A (45).

The reconstituted fusion protein [aFGF-dtA+B], which is able to translocate into cells containing diphtheria toxin receptors, stimulated DNA synthesis both in untransfected U2OS Dr1 cells and in transfected U2OS Dr1 R4 cells but had little effect on NIH 3T3 cells, which lack diphtheria toxin receptors (Fig. 6c).

The stimulation of DNA synthesis in U2OS Dr1 cells by [aFGF-dtA+B] was equally as sensitive to aphidicolin as was DNA synthesis stimulated by either 1 or 10% FCS (~50 ng/ml reduced the incorporation of [^{3}H]thymidine to half in each case [data not shown]). This indicates that [aFGF-dtA+B]-induced [^{3}H]thymidine incorporation represents replicative DNA synthesis (21, 53).

Whereas aFGF-dtA (like aFGF alone) was not able to stimulate [^{3}H]thymidine incorporation in untransfected U2OS Dr1 cells, reconstituted [aFGF-dtA+B] stimulated incorporation in a dose-dependent manner (Fig. 6d). Heparin increased the ability of aFGF to stimulate [^{3}H]thymidine incorporation in U2OS Dr1 R4 cells as well as in NIH 3T3 cells (Fig. 6e), whereas the stimulation by [aFGF-dtA+B] in U2OS Dr1 R4 cells was strongly inhibited by heparin (Fig. 6), which renders it translocation incompetent (63). The construct [aFGF-dtA-SS+B], which was found to be translocation incompetent (Fig. 5) because of a disulfide bond introduced into dtA (15), was also unable to stimulate [^{3}H]thymidine incorporation in U2OS Dr1 R4 cells (Fig. 6e). It should be noted that the background level of [^{3}H]thymidine incorporation varied somewhat between experiments (e.g., compare Fig. 6c and d).

To test the possibility that the stimulatory effect of [aFGF-dtA+B] in U2OS Dr1 cells was due to small amounts of FGF receptors not recognized in our binding studies, we carried out experiments with U2OS Dr1 cells transfected with a deletion mutant of FGFR4 that lacked most of the cytoplasmic domain, including the kinase domain. Such molecules have been described as dominant negative because of their ability to form dimers with normal receptors and inability to induce cross-phosphorylation (1, 28, 32). The results in Fig. 7a show that U2OS Dr1 cells transfected with this mutant bound aFGF in a way similar to that of cells transfected with the wild-type receptor (Fig. 2a). Cross-linking experiments with ^{125}I-aFGF showed a band with a molecular mass corresponding to ~70 kDa in transfected cells, not in untransfected cells (data not shown). This corresponds to a complex of the deletion mutant of the receptor (molecular mass, ~55 kDa) and aFGF (molecular mass, 16 kDa).

As expected, neither aFGF nor aFGF-dtA stimulated DNA synthesis in these cells (Fig. 7c). On the other hand, [aFGF-dtA+B] stimulated DNA synthesis in cells transfected with the deletion mutant to an extent similar to that in untransfected U2OS Dr1 cells (Fig. 7b and c). It is therefore unlikely that [aFGF-dtA+B] stimulates DNA synthesis by acting on FGF receptors.

Although [aFGF-dtA+B] stimulated DNA synthesis in Vero Dr22 and U2OS Dr1 cells, it was unable to stimulate proliferation in these cells (62). This could have been due to a requirement for a second signal, as provided by receptor-mediated tyrosine phosphorylation. If this is the case, [aFGF-dtA+B] should stimulate proliferation in transfected cells, because in this case two signals would be transmitted, one due to the binding of the construct to FGFR4 and induction of tyrosine kinase and the other one due to translocation of aFGF-dtA to the cytosol with subsequent transport to the nucleus and stimulation of DNA synthesis. The data in Fig. 8a and c show that proliferation in transfected cells was stimulated not only by aFGF but also by [aFGF-dtA+B]. It should be noted that heparin, which increased the stimulating activity of aFGF, had little effect on aFGF-dtA and completely blocked the stimulating effect of [aFGF-dtA+B], in accordance with the finding in Fig. 5 that heparin prevented the translocation of this fusion protein into cells. In NIH 3T3 cells, only aFGF (with or without heparin), not [aFGF-dtA+B], induced cell proliferation, in accordance with the finding that this fusion protein is unable to enter these cells, which lack diphtheria toxin receptors. In untransfected U2OS Dr1 cells, neither aFGF, aFGF-dtA, nor [aFGF-dtA+B] stimulated proliferation (Fig. 8b).

In untransfected cells, [aFGF-dtA+B] stimulated [^{3}H]thy-

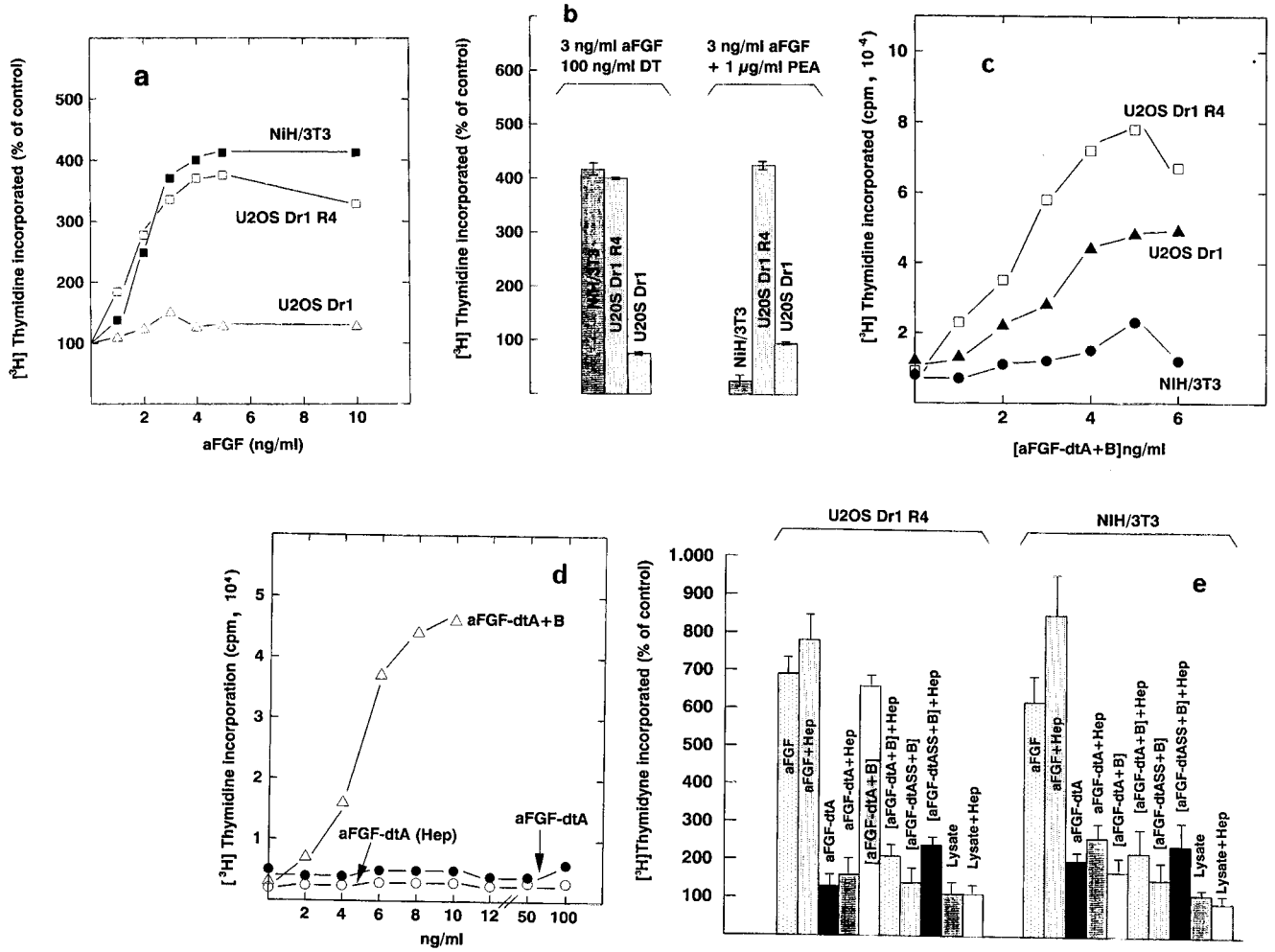

FIG. 6. Abilities of different constructs to stimulate DNA synthesis in cells with and without receptors for aFGF and diphtheria toxin (DT). (a) Cells growing on 24-well microtiter plates (10^5 cells per well) were preincubated for 48 h in serum-free medium at 37°C. Then cells were treated with increasing amounts of aFGF, and the incubation was continued for 24 h at 37°C. During the last 6 h, cells were labelled with [^{3}H]thymidine as previously described (23), and the incorporated radioactivity was measured. (b) Cells were incubated, as described for panel a, with 5 ng of aFGF per ml and 100 ng of DT per ml or 1 μg of *Pseudomonas* exotoxin A (PEA) per ml. Bars represent the ranges of duplicate experiments. (c) As indicated, to cells were added increasing amounts of [aFGF-dtA+B], and the cells were incubated for 24 h (NIH 3T3) or 48 h (U20S Dr1 and U20S Dr1 R4). (d) Increasing amounts of constructs, as indicated, were added to untransfected U2OS Dr1 cells, which were then treated as described for panel c. (e) Different constructs (5 ng/ml) were added to cells, as indicated. In some cases, 15 U of heparin (Hep) per ml was also added. Cells were then treated as described for panel a. In the case of U2OS Dr1 R4 cells treated with [aFGF-dtA+B], the incubation period was 48 h. Bars represent the ranges of duplicate experiments. The experiment was repeated six times.

midine incorporation as a wave, with a maximum at 48 h in U2OS Dr1 cells (Fig. 8d), in accordance with our previous findings (62). In transfected cells, [^{3}H]thymidine incorporation continued to increase throughout the 3 days of observation. This suggests that in untransfected cells, DNA synthesis largely stopped after passing through the S phase once, whereas transfected cells were capable of passing through mitosis and starting new rounds of DNA synthesis.

DISCUSSION

The main findings reported here are that DNA synthesis was stimulated only under conditions in which aFGF or aFGF-dtA was found in the nuclear fraction and that proliferation was observed only in cells in which both tyrosine phosphorylation and nuclear targeting occurred. Our interpretation of these data is that two signals are necessary to stimulate cell proliferation in U2OS Dr1 R4 cells.

As shown previously (62, 68, 69) with NIH 3T3 and BALB/c 3T3 cells incubated with labelled aFGF, part of the cell-associated growth factor was found in the nuclear fraction (i.e., the detergent-insoluble fraction, containing nuclei and cytoskeletal material). The present data demonstrate that this was also the case with U2OS Dr1 R4 cells. On the other hand, in the case of U2OS Dr1 cells, with which binding appears to occur to surface heparans, aFGF was recovered only from the cytoplasmic fraction (which, in addition to cytosol, consists of dissolved membranes and the contents of intracellular vesicles) and no labelled material could be recovered from the nuclear fraction. Only when aFGF was fused with dtA and reconstituted with dtB was it found in the nuclear fraction of U2OS Dr1 cells, which lack FGF receptors. Specific FGF receptors therefore appear to facilitate translocation of aFGF across cellular membranes.

The ability of [aFGF-dtA+B] to stimulate DNA synthesis was inhibited by heparin, which induces tight folding of aFGF

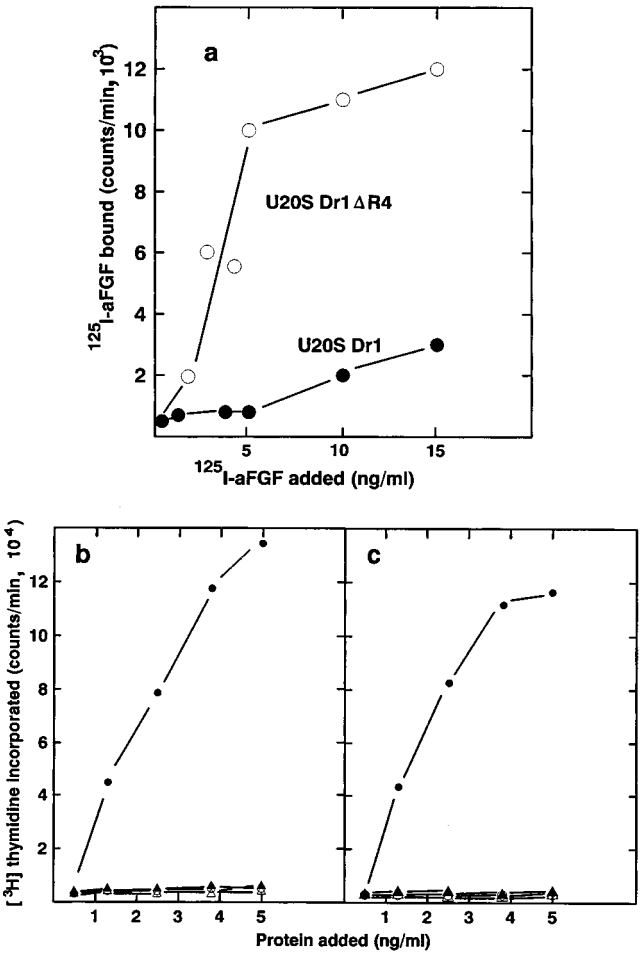

FIG. 7. Ability of U2OS Dr1 cells transfected with mutant FGFΔR4 to bind aFGF (a) and respond with DNA synthesis to various compounds (b and c). (a) Increasing amounts of ^{125}I-aFGF were added to U2OS Dr1 and U2OS Dr1 ΔR4 cells growing on 24-well microtiter plates coated with gelatin. After 4 h at 4°C, cells were washed and dissolved in 0.5 M NaOH, and the cell-associated radioactivity was measured. Serum-starved U2OS Dr1 (b) and U2OS Dr1 ΔR4 (c) cells were treated with increasing concentrations of various compounds, and then their abilities to incorporate [^{3}H]thymidine during 6 h were measured. ●, [aFGF-dtA+B]; ○, [aFGF-dtA+B] and 10 U of heparin per ml; ▲, aFGF and 10 U of heparin per ml; △, aFGF-dtA.

and inhibits translocation of aFGF-dtA into the cell (63). It was recently reported that heparin can activate FGF receptors and stimulate cell proliferation in a lymphoid cell line in the absence of growth factor (20). We did not see any stimulatory effect of heparin alone in our cell lines.

We also considered the possibilities that [aFGF-dtA+B] could be a more potent ligand than aFGF alone in stimulating a small number of (undetected) endogenous FGF receptors on U2OS Dr1 cells and that the observed stimulation of DNA synthesis could be due to receptor binding rather than to translocation of aFGF-dtA to the cytosol. To test this, we carried out experiments with [aFGF-dtA-SS+B], which is translocation incompetent (62, 63) but otherwise similar to [aFGF-dtA+B]. There was no stimulation of DNA synthesis or cell proliferation with this construct.

To further test this possibility, we also carried out experiments with U2OS Dr1 cells transfected with a deletion mutant of FGFR4 lacking most of the cytoplasmic part, including the whole kinase domain. Such receptor mutants have been shown to act as dominant negative receptors (1, 28, 32). We found that [aFGF-dtA+B] induced [^{3}H]thymidine incorporation in these cells to the same extent as that in untransfected U2OS Dr1 cells. This further supports the notion that the activation of endogenous FGF receptors by [aFGF-dtA+B] is not the reason for the stimulation of DNA synthesis.

We also tested the ability of aphidicolin to inhibit the DNA synthesis induced by [aFGF-dtA+B] in untransfected U2OS Dr1 cells. At concentrations between 20 ng/ml and 1 μg/ml, aphidicolin is described to inhibit replicative DNA synthesis by polymerases α and δ, whereas more than 2 μg/ml is required to inhibit repair synthesis (21, 53). The present finding that the DNA synthesis induced by [aFGF-dtA+B] is 50% inhibited by 50 ng of aphidicolin per ml supports our previous conclusion (62) that replicative DNA synthesis is stimulated by this construct.

Both aFGF and basic FGF are synthesized without a signal sequence, and they are present in the cytosol and nucleus of a cell producing them (8, 10, 35). So far, no defined intracellular function of FGF has been described, and their presence in producing cells has been considered to be that of molecules waiting for release, either by cell damage, which could play a role in wound healing (39, 66) and the formation of atherosclerotic plaques (7, 13), or by some kind of specific export mechanism (19, 38).

It is not clear what role aFGF plays in the nucleus. It has been reported that basic FGF enters the nucleolus and induces the transcription of ribosomal genes (5). aFGF was also recently localized to the nucleoli of pulmonary artery endothelial cells (36). One form of FGF3 was found to enter the nucleus and associate with the nucleolus, although it inhibited rather than stimulated DNA synthesis (29).

The expression of basic FGF in baby hamster kidney-derived cells (40) and aFGF in Swiss 3T3 cells (25) resulted in autonomous growth and tumorigenesis under conditions in which the growth factor could not be detected extracellularly and the addition of antibodies to the growth factor to the medium did not inhibit the effect. Our observation that aFGF translocated into cells as a fusion protein with diphtheria toxin stimulates DNA synthesis suggests that in cells producing the growth factor, it may also have an intracellular role to play. In fact, the amount of basic FGF found in growth factor-producing bovine aortic endothelial cells (2) is similar to the amount of aFGF-dtA translocated into Vero Dr22 and U2OS Dr1 cells, which are rich in diphtheria toxin receptors. On the other hand, in HeLa Dr1 cells (which have considerably fewer diphtheria toxin receptors), we were unable to translocate into cells measurable amounts of the fusion protein and there was no stimulation of DNA synthesis (62). The reason for this could be that a certain intracellular concentration of the growth factor must build up before DNA synthesis is stimulated and that this level is not reached in HeLa Dr1 cells. Our previous finding (62) that [^{3}H]thymidine incorporation is stimulated earlier in Vero Dr22 cells than in U2OS Dr1 cells, which contain fewer diphtheria toxin receptors than do Vero Dr22 cells, agrees with this interpretation.

Although both receptor activation and internalization of the growth factor appear to be required for proliferation of the cells studied here, it is possible that in some cells one signal is sufficient. Thus, in cells expressing aFGF which remains intracellular, only receptor activation may be required.

While aFGF stimulated DNA synthesis and proliferation in U2OS Dr1 cells transfected with FGFR4, Wang et al. (61) found no stimulation in murine lymphoid cells and rat myoblasts transfected with FGFR4. Shaoul et al. (52) found lower stimulation of proliferation in FGFR4-transfected rat myo-

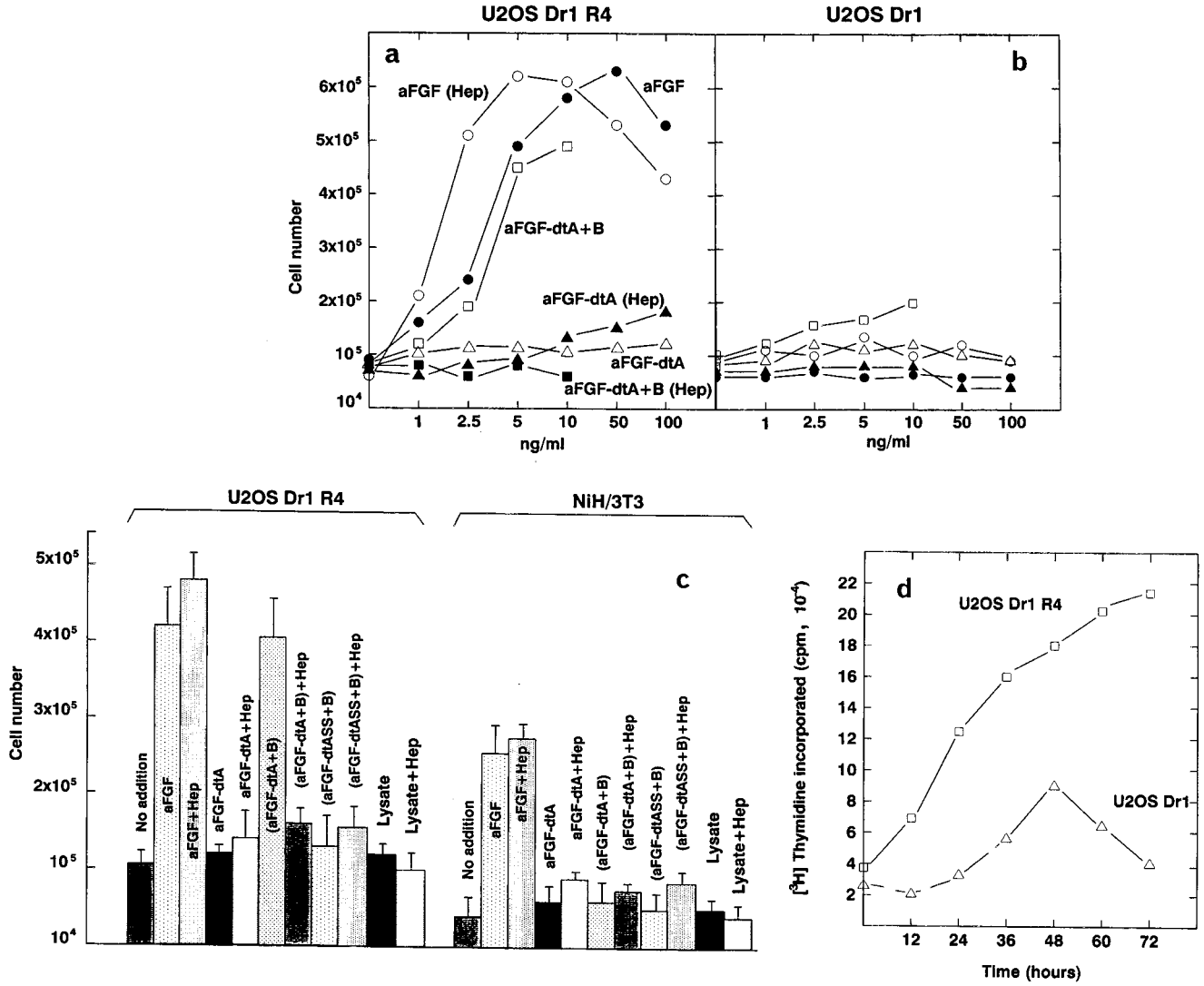

FIG. 8. Abilities of different constructs to stimulate cell proliferation. As indicated, cells were serum starved for 48 h and then treated with increasing amounts (a and b) or 5 ng (c) of either aFGF, aFGF-dtA, [aFGF-dtA+B], or [aFGF-dtA-SS+B] in the absence or presence of 15 U of heparin (Hep) per ml. The incubation was continued for 5 days with a change of medium on the third day. Finally, cells were detached from the plastic with trypsin and the cell number was determined. Bars indicate the ranges of duplicate parallel samples. The experiment was repeated six times. (d) To cells that were serum starved for 72 h was added 5 ng of [aFGF-dtA+B] per ml. After the periods indicated, the abilities of cells to incorporate [^{3}H]thymidine during 6 h were measured.

blasts than in myoblasts transfected with FGFR1. Vainikka et al. (59) found the stimulation of DNA synthesis in rat myoblasts transfected with FGFR4 to be almost as strong as that when these cells were transfected with FGFR1. All of these authors found tyrosine phosphorylation of the receptor and MAP kinase (or ERK). Wang et al. (61) found no induction of c-*fos* and *tis11* mRNA expression. We also did not find the induction of c-*fos* by aFGF in FGFR4-transfected cells, while we saw strong induction in NIH 3T3 cells, which contain FGFR1 and FGFR2. Possibly, the inability to induce c-*fos* distinguishes FGFR4 from other FGF receptors.

An increasing number of cytokines and growth factors appear to be transported to the nucleus after being added externally to cells. Basic FGF (5, 55), interleukin 1 (54), angiogenin (36), and Schwannoma-derived growth factor (30) contain putative nuclear localization sequences and have been either recovered from the nuclear fraction of lysed cells or visualized in the nuclear region morphologically. It is an interesting possibility that these and other growth factors employ a dual mode of signal transduction.

ACKNOWLEDGMENTS

We are grateful to K. Sandvig and M. Lanzrein for critically reading the manuscript. The expert work of Jorunn Jacobsen in the transfection, selection, and maintenance of cell lines is gratefully acknowledged.

This work was supported by the Norwegian Cancer Society, the Novo Nordisk Foundation, the Norwegian Research Council for Science and Humanities, the Blix Legat, Rachel and Otto Kr. Bruun's Legat, and the Jahre Foundation. R.M. has a Personal Investigator Fellowship from the Spanish Ministry of Education and Science. O.K. and A.R. are Fellows of the Norwegian Cancer Society.

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