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The Receptor Tyrosine Kinase ARK Mediates Cell Aggregation by Homophilic Binding

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The ARK (AXL, UFO) receptor is a member of a new family of receptor tyrosine kinases whose extracellular domain contains a combination of fibronectin type III and immunoglobulin motifs similar to those found in many cell adhesion molecules. ARK mRNA is expressed at high levels in the mouse brain, prevalently in the hippocampus and cerebellum, and this pattern of expression resembles that of adhesion molecules that are capable of promoting cell aggregation through homophilic or heterophilic binding. We report here the ability of the murine ARK receptor to mediate homophilic binding. Expression of the ARK protein in *Drosophila* S2 cells induces formation of cell aggregates consisting of ARK-expressing cells, and aggregation leads to receptor activation, with an increase in receptor phosphorylation. Homophilic binding does not require ARK tyrosine kinase activity, since S2 cells expressing a receptor in which the intracellular domain was deleted were able to undergo aggregation as well as cells expressing the wild-type ARK receptor. Similar results were obtained with NIH 3T3 and CHO cells expressing high levels of ARK, although in this case ARK expression appeared to be accompanied by constitutive activation. The purified recombinant extracellular domain of ARK can induce homotypic aggregation of coated fluorescent beads (Covaspheres), and this protein can also function as a substrate for adhesion by S2 and NIH 3T3 cells expressing ARK. These results suggest that ARK represents a new cell adhesion molecule that through its homophilic interaction may regulate cellular functions during cell recognition.

The mechanisms by which cells undergo growth and differentiation are regulated by the phosphorylation of proteins that are activated by signals generated in the extracellular compartment. Receptor tyrosine kinases (RTKs) constitute a large class of proteins which are responsible for the transduction of the signal through the cell membrane. An activated receptor can rapidly associate with the SH2 domains of cellular proteins, like GRB2, phospholipase C-γ, GTPase-activating protein, or phosphatidylinositol 3-kinase and trigger second-messenger cascades which lead to cell division or differentiation (32).

The murine tyrosine kinase receptor ARK (adhesion-related kinase) was isolated, in our laboratory, by using a strategy aimed at identifying novel tyrosine kinases (30). It is a transmembrane protein of 888 amino acids whose extracellular domain contains a combination of two immunoglobulin G (IgG)-like domains and two segments homologous to fibronectin (FN) type III repeats and thus resembles the extracellular domains found in many cell adhesion molecules (CAMs) (40). The ARK cytoplasmic domain has the typical structure of a tyrosine kinase, with a short intervening sequence, similar to that found in the insulin and fibroblast growth factor receptors (FGFRs) (15). The human homolog of ARK (which has been called AXL or UFO) was also independently isolated by two other laboratories from the DNA of patients with chronic myelogenous leukemia by using a tumorigenicity assay (18, 27). Recently several cDNAs encoding new members of this family of RTKs have been isolated in different laboratories. These proteins have been variously designated Tyro 3 (23), Sky (28), Rse (25), or Brt (12), and their expression appears to be predominantly localized to the central nervous system.

Most of the integral membrane proteins involved in cell adhesion can be classified in three structural families (19): the immunoglobulin superfamily, which can mediate cell-cell interaction, generally in a Ca^{2+}-independent manner (40); cadherins, which homophilically mediate binding in a Ca^{2+}-dependent manner (36, 37); and integrins, which can interact, in a Ca^{2+}- or Mg^{2+}-dependent manner, with components of the extracellular matrix or with counterreceptors of the immunoglobulin superfamily on the cell surface (17). Members of the immunoglobulin superfamily are known to have a wide range of biological functions and to participate in homophilic and heterophilic interactions (1, 40). The presence of conserved IgG(C)-like domains in their extracellular domain has been shown for many of these proteins and is required to promote intercellular adhesion, while the FN repeats maybe dispensable (10). Alternative mRNA splicing and posttranslational modifications can lead to different forms of CAMs (16). These may have a transmembrane form, be associated with the cell membrane through a lipid anchor, or be secreted by the cells (6, 13). While N-CAM is known to play a role during the development of the central nervous system (6, 7), other molecules like ICAM-1 and V-CAM-1 are involved in inflammation and wound healing (8); PECAM-1/CD31, which was isolated as a platelet/endothelial CAM, is also expressed at intercellular junctions (2, 26). The carcinoembryonic antigen has been found to be expressed in tumors as well as in normal tissues (3).

The structure of the ARK protein is interesting in that it appears to represent a link between the better-known CAMs, which are not tyrosine kinases, and RTKs. It is therefore tempting to speculate that it may be involved in sending signals to the cell nucleus that originate from cell-to-cell contact as a

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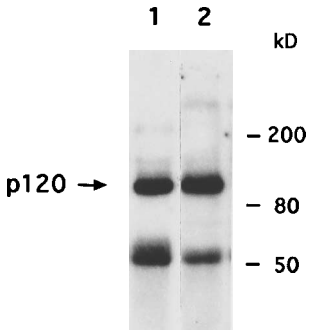

FIG. 1. Expression and phosphorylation of ARK in lysates of NIH 3T3 cells. Lysates of NIH 3T3 cells were incubated with polyclonal antibodies raised against the extracellular domain of the ARK receptor. Immunoprecipitates were separated by SDS-PAGE (10% gel), subjected to Western blotting analysis using anti-ARK (lane 1) or antiphosphotyrosine (lane 2) antibodies, incubated with ^{125}I-labeled protein A, and autoradiographed. The migration of the ARK protein (p120) is indicated on the left. The lower band corresponds to the 50-kDa heavy chain of immunoglobulins that is recognized by protein A. Molecular weight markers are indicated.

result of a homophilic or heterophilic interaction with membrane-associated proteins. Homophilic interaction of a molecule with tyrosine kinase activity has recently been shown for the *Drosophila* protein D-TRK (29). More recently, two receptor tyrosine phosphatases, R-PTP-κ and R-PTP-μ (4, 31), also members of the immunoglobulin superfamily, have been shown to promote cell adhesion through a homophilic mechanism.

We report here that the ARK receptor can mediate cell-cell interactions through homophilic binding. Expression of the *ark* cDNA in *Drosophila* S2 cells induces aggregation which is accompanied by increased receptor phosphorylation. Adhesion requires only the extracellular domain of ARK, and in vitro experiments show that the ARK extracellular domain can induce homotypic aggregation of coated fluorescent beads (Covaspheres) in a Ca^{2+}-independent manner. This recombinant protein can also function as a substrate for adhesion by cells overexpressing the ARK receptor. Although the biological role of ARK remains unknown, these results suggest that this RTK represents a new CAM whose homophilic interaction regulates cellular functions during cell-to-cell contact.

MATERIALS AND METHODS

Plasmids. The 4.1-kb *ark* cDNA was introduced in both orientations into the *Eco*RI site of the *Drosophila* pCasPer vector containing the heat shock protein 70 (*hsp70*) promoter (39). The 1.6-kb fragment encoding a truncated tyrosine kinase receptor (ARK-TKΔ), in which an oligonucleotide carrying a stop codon was inserted immediately downstream of the codon for amino acid 522, was cloned in the pCasPer vector. The 4.5-kb fragment corresponding to the *bek-ark* cDNA, in which the extracellular and transmembrane domains of *ark* cDNA were substituted with the 2.0-kb fragment of the BEK receptor cDNA (24), was introduced into the *Eco*RI site of the *Drosophila* pRmHa-3 vector under the control of the metallothionein promoter (5). The 4.1-kb *ark* cDNA and the 4.5-kb *bek-ark* cDNA were introduced into the *Eco*RI site of the p91023(B) mammalian expression vector (41) and used for transfection of CHO-DG44 cells. The cDNA fragment encoding the secreted extracellular domain of the ARK protein (ECD-AP) (amino acids 1 to 437) was fused in frame with the human placental alkaline phosphatase gene, introduced into the APtag vector (11), and used for transfection of NIH 3T3 cells. A plasmid encoding a secreted alkaline phosphatase protein (SE-AP), kindly provided by J. Sap (Department of Pharmacology, NYU Medical Center), was generated by cloning a signal peptide sequence in frame with the alkaline phosphatase gene. The 1.6-kb fragment encoding the truncated ARK-TKΔ receptor was cloned into the AP tag vector after deletion of the sequences encoding the alkaline phosphatase gene.

Culture and transfection of *Drosophila* S2 cells. S2 cells (33) were grown at room temperature in Schneider medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and 2 mM glutamine. Cells were transfected with DNA-calcium phosphate precipitates, using 16 μg of each plasmid cotransfected with 4 μg of plasmid pPC4, which carried the α-amanitin resistance gene (20). Pools of stably transfected cells were selected in the presence of 5 μg of α-amanitin per ml for 30 days.

Culture and transfection of mammalian cells. CHO-DG44 cells, which are dihydrofolate reductase negative, were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum, 0.1 mM hypoxanthine, 0.02 mM thymidine, and 10 mM proline. Transfection was performed by the calcium phosphate procedure, using 5 μg of plasmids. Positive clones were selected in medium lacking hypoxanthine and thymidine. The level of expression in transfected CHO-ARK and CHO-BEK-ARK clones was increased by using methotrexate (50 μM), and the level of amplification was analyzed by Northern (RNA) blotting. NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Cells were cotransfected by using 5 μg of plasmids with 100 ng of a plasmid carrying the neomycin resistance gene. Positive clones were selected with 400 μg of Geneticin (G418; Gibco BRL) per ml.

Antibodies. Rabbit polyclonal antiserum directed against the N terminus of the receptor was raised by using the 115-kDa affinity-purified protein ECD-AP (see above). Fab′ fragments were generated by using papain agarose beads (Pierce). The IgG fraction was purified by using an Immunopure (A/G) IgG purification kit (Pierce). Polyclonal antiserum against the C terminus of the receptor was produced by using a fusion protein between TrpE (22) and the sequence encoding amino acids 791 to 888 of the ARK protein. The polyclonal antibody directed against phosphotyrosine was provided by Ben Margolis (Department of Pharmacology, NYU Medical Center). The monoclonal antibody against human placental alkaline phosphatase was from Medix Biotech.

Electrophoresis and immunoblotting. Cells were lysed with Triton buffer (10 mM phosphate buffer [pH 7.4]; 100 mM NaCl, 1% Triton X-100, 5 mM EDTA) containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 4 μg of aprotinin per ml and incubated with antibodies immobilized on protein-A Sepharose. Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose. Filters were saturated with a solution of 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) and incubated with anti-ARK antiserum (diluted 1:100 in 5% BSA–TBS) or with antiphosphotyrosine antiserum (diluted 1:200 in 5% BSA–TBS). Proteins were visualized by autoradiography after incubation with ^{125}I-protein A (10 mCi/mg; NEN-Dupont).

Fluorescence-activated cell sorting (FACS) analysis. S2 cells transfected with the wild-type *ark* cDNA in the pCasPer vector were heat shocked for 30 min at 37°C to induce ARK expression and allowed to recover for 90 min at room temperature. The cells were washed with ice-cold buffered salt solution (BSS) (35) and incubated with IgGs purified from an antiserum that recognizes the N terminus of the receptor. IgGs were previously absorbed to untransfected S2 cells in order to avoid nonspecific binding and added to the cells at the concentration of 50 μg/ml on ice for 30 min. The primary antibody was then removed, and the cells were washed with ice-cold BSS and fixed with 4% paraformaldehyde on ice for 20 min. After being washed with BSS, the cells were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody (Molecular Probes, Inc., Eugene, Oreg.) for 30 min at 4°C and examined with a FACScan flow cytometer (Becton Dickinson, Lincoln Park, N.J.).

Aggregation assays. S2 cells transfected with the pCasPer vector were induced by heat shock for 30 min at 37°C in order to induce expression of the proteins and allowed to recover for 90 min at room temperature. S2 cells transfected with the pmHa-3 vector were induced by adding $CuSO_4$ to 0.7 mM for 18 h. Adherent cells were washed with BSS, collected, and resuspended in BSS at the concentration of 4×10^6 cells per ml. An aggregation assay was performed in Coulter Counter vials at room temperature on a rotary shaker at 90 rpm. Quantitation of aggregate formation was monitored at intervals in a Coulter Counter. Settings were as follows: lower threshold, 25; upper threshold, 100; current, 500 mA; amplification, 4. Since aggregation leads to a decrease of the total number of particles in suspension, the extent of aggregation was estimated by dividing the number of particles at time t by the number at time zero. Aggregates were also visualized with an inverted microscope. For experiments involving the fluorescence dye DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Inc.), the dye was added to the medium during heat shock at the final concentration of 5 μM. Cells were washed extensively with BSS, and 2×10^6 cells of each population were mixed and allowed to coaggregate in a volume of 1 ml. Aggregate composition was monitored under visible and fluorescence microscopy. Aggregation experiments using mammalian cells were performed as follows: Cells at 80 to 90% confluence were washed with phosphate-buffered saline (PBS) and incubated with 0.0025% trypsin for 3 to 4 min at room temperature. The trypsin was removed, and the cells were resuspended in S-MEM (GIBCO) containing 10% dialyzed calf serum. After three passages through an 18-gauge syringe, cells were diluted at the final concentration of 1×10^6 to 2×10^6/ml and allowed to aggregate at 37°C on a rotary shaker at 90 to 100 rpm. Samples were removed at intervals and counted in a Coulter Counter, using the same settings described for S2 cells but with an amplification of 8.

Purification of soluble proteins. The 115-kDa ECD-AP fusion protein and the relative 67-kDa control protein SE-AP were produced as secreted proteins in NIH 3T3 cells. Both proteins were affinity purified from the supernatant of transfected cells, using an anti-alkaline phosphatase monoclonal antibody (Medix Biotech) column. The proteins were eluted with PBS containing 50% ethyl-

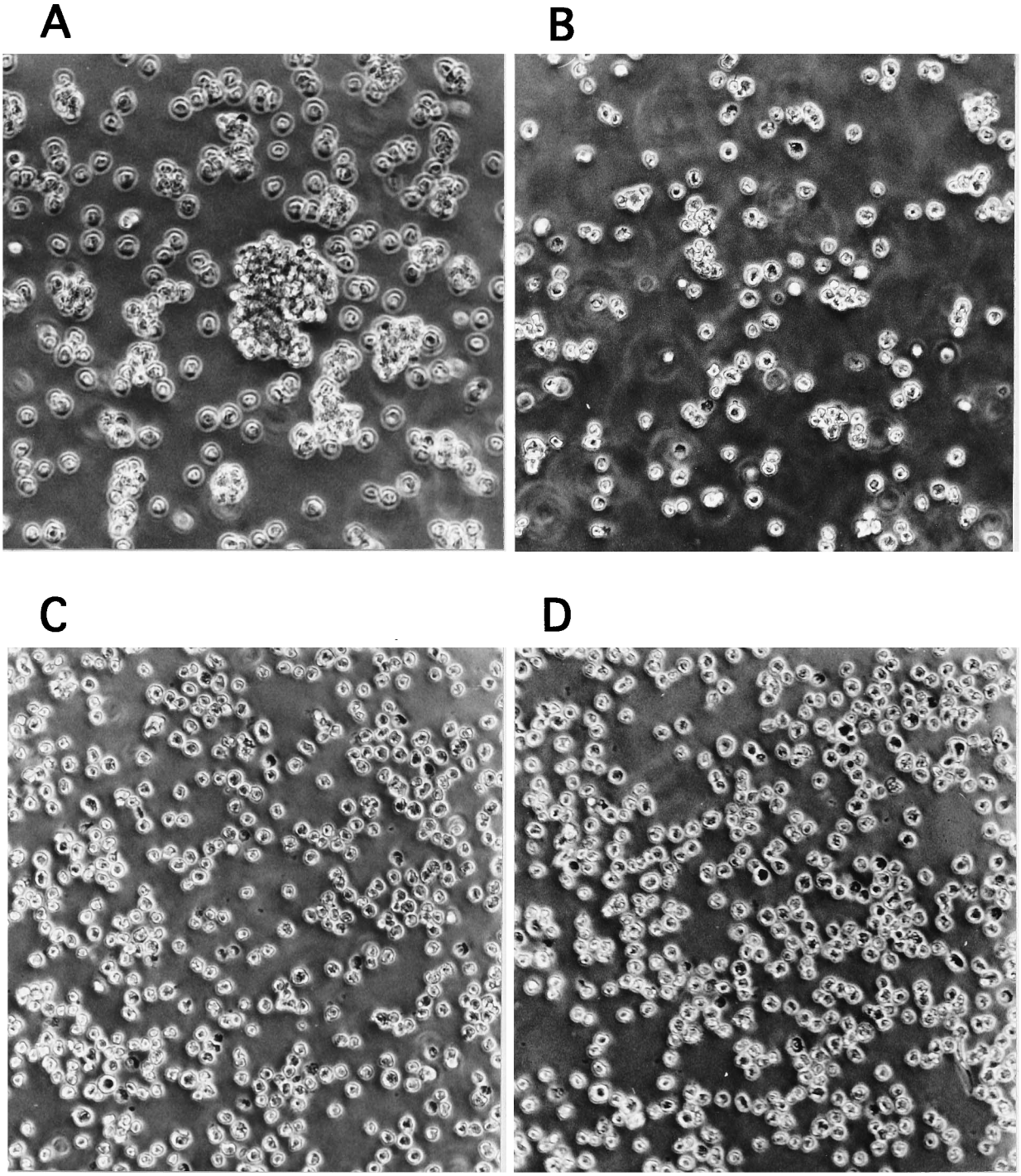

FIG. 2. Aggregation induced by the ARK protein in S2 cells. Transfected S2 cells were induced as described in Materials and Methods and resuspended in BSS at the concentration of 4×10^6 cells per ml. Cells then were allowed to aggregate for 1 h at room temperature with rotary shaking (90 rpm). Photographs of S2 cells after induction and aggregation for 1 h are shown. (A) ARK-expressing S2 cells. (B) ARK-expressing S2 cells in which aggregation was performed in the presence of a 100-μg/ml concentration of Fab′ fragment raised against the extracellular domain of ARK. (C) S2 cells expressing the chimeric receptor BEK-ARK. (D) Control S2 cells transfected with the *ark* cDNA in the antisense orientation.

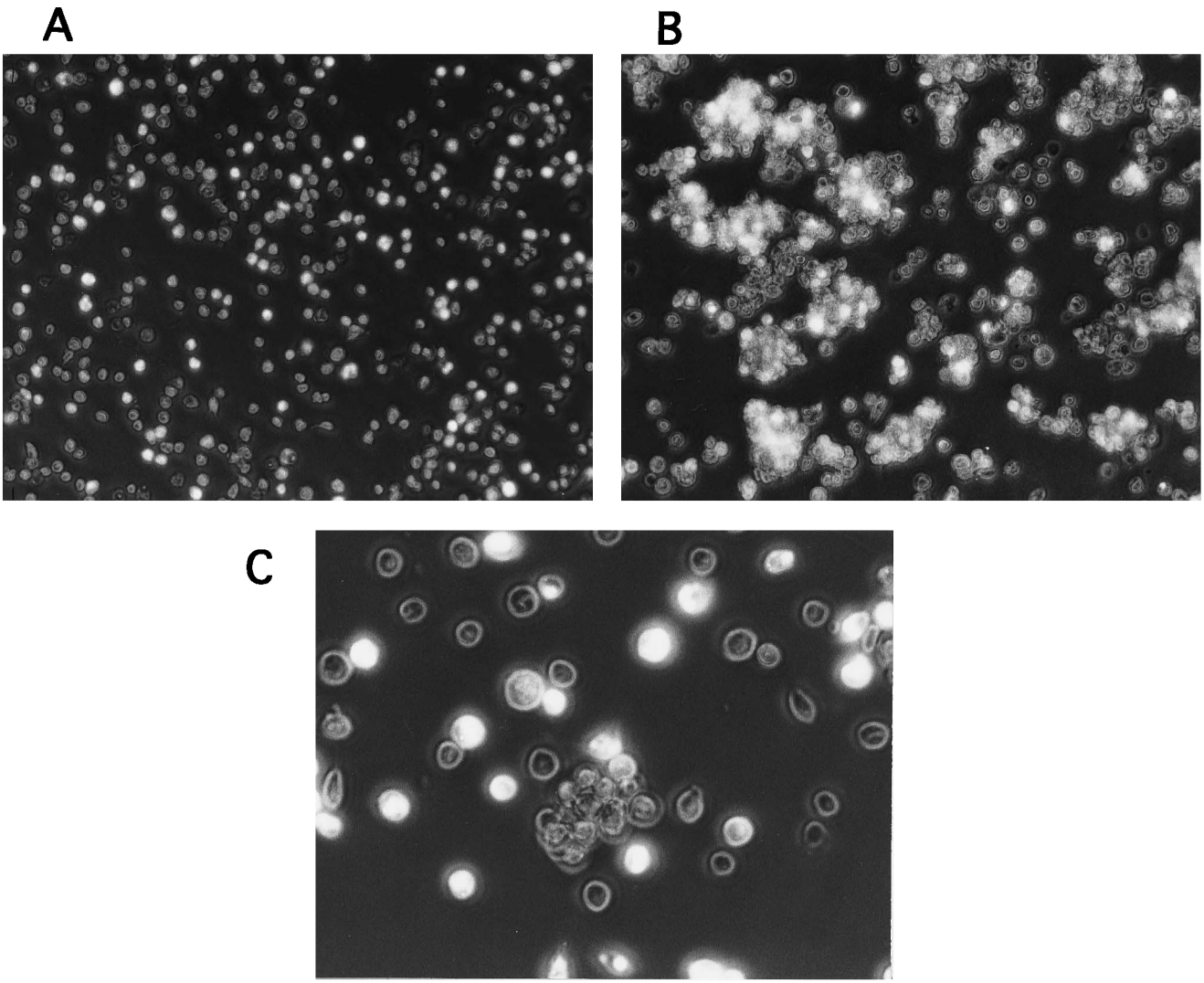

FIG. 3. Aggregates consist of cells expressing the ARK receptor. Different populations of transfected S2 cells were labeled with the fluorescent dye DiI during induction. After recovery for 90 min at room temperature, equal numbers of labeled and unlabeled cells were allowed to aggregate for 1 h under standard conditions. The resulting aggregates were visualized under visible and fluorescence microscopy. Labeled cells are white in the photographs. (A) Mixture of labeled and unlabeled control S2 cells (transfected with the *ark* cDNA in the antisense orientation). (B) Unlabeled ARK expressing cells that were allowed to aggregate with labeled ARK-expressing cells. (C) Higher magnification of the aggregates in which unlabeled ARK-expressing cells were mixed with labeled control cells.

ene glycol and dialyzed extensively against PBS. Protein concentration and enzymatic activity were estimated as described previously (11). The purity of these protein preparations was estimated to be about 30%.

Covasphere aggregation assay. Fifty microliters of green-fluorescing Covasphere beads (0.5-μm diameter; Duke Scientific, Palo Alto, Calif.) was incubated with 10 μg of ECD-AP protein or control proteins and 3.3 μg of SE-AP protein in 100 μl. After coupling for 1 h at 37°C and then 1 h at 4°C, samples were sonicated for 15 s and washed with blocking buffer (10 mM Tris [pH 7.4], 1 mg of BSA per ml) for 30 min on ice. For the aggregation assay, Covaspheres were resonicated and resuspended in 10 times the original volume (50 μl) of PBS. The number of aggregated Covaspheres was counted with a Coulter Counter, using a setting that detects particles larger than approximately 60 Covaspheres (14).

Adhesion assay. For adhesion assays, 3-μl aliquots of protein samples (100 μg/ml in the case of ECD-AP and the control proteins and 33 μg/ml for SE-AP protein) were spotted on 35-mm-diameter bacteriological petri dishes and allowed to bind for 30 min at room temperature. This yielded circular protein-coated areas approximately 3 mm in diameter. After aspiration of the solutions, the surface of the dishes was saturated with 1% heat-inactivated BSA (Sigma) for 60 min at 37°C. The plates were incubated with S2 cells (4×10^6/ml) in BSS with rotary shaking (50 rpm) for 1 h. NIH 3T3 cells were washed in PBS and incubated with 0.0025% trypsin at room temperature. After 3 to 4 min, trypsin was removed and the cells were resuspended in S-MEM–0.1% serum at the concentration of 10^6/ml. Cells were allowed to attach to the coated petri dishes for 1 h at 37°C without shaking. The plates were then washed with PBS, and the cells attached were fixed in 3.7% formaldehyde stained with crystal violet and counted under a microscope.

In situ hybridization. The brain from an adult nude mouse was embedded in Tissue-tek optimal-cutting-temperature compound. Serial frozen sections of 4 μm were collected onto poly-L-lysine-coated slides; after dehydration, the slides were fixed for 20 min at room temperature in 4% paraformaldehyde in PBS, rinsed in PBS, and dehydrated. Plasmid pB4 containing a 446-bp *Eco*RI-*Pst*I fragment of the mouse *ark* cDNA, encoding amino acids 106 to 252, was used as the template for the antisense and sense riboprobes. ^{35}S-labeled probes ([α-^{35}S]UTP; >1,000 mCi/mmol) were synthesized by using an RNA transcription kit (Boehringer). Hybridization was performed as described previously (38).

RESULTS

ARK is a tyrosine kinase and is constitutively phosphorylated in NIH 3T3 and CHO cells. As previously described (30), NIH 3T3 cells express ARK mRNA, and similar results were obtained with CHO cells, although the level of expression was somewhat lower. Using an antiserum that recognizes the extracellular portion of the receptor, we were able to immuno-

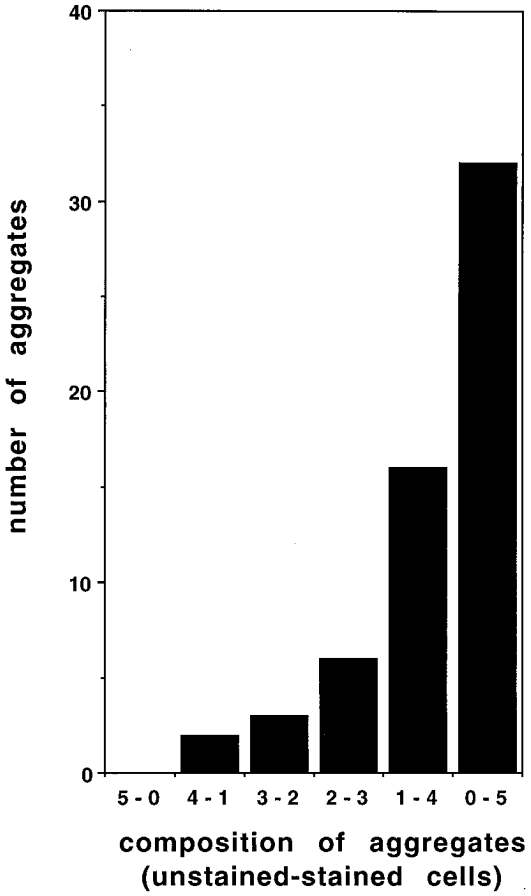

FIG. 4. Frequency distribution of mixed aggregates. Mixed aggregation assays were performed with equal numbers of untransfected S2 cells (unstained) and S2 cells transfected with the *ark* cDNA labeled with DiI (stained). The cells were stained during the induction and assayed for aggregation as described in Materials and Methods. The number of stained and unstained cells within each five-cell aggregate was counted by using an inverted microscope. Twenty microscopic fields were counted, and analysis was performed as described by Sieber and Roseman (34).

precipitate from NIH 3T3 lysates a band of approximately 120 kDa corresponding to the ARK receptor (Fig. 1). The murine ARK protein could also be identified in CHO-DG44 cells transfected with the 91023(B) vector containing the *ark* cDNA (not shown).

To verify ARK kinase activity, we performed in vitro kinase assays with [γ-^{32}P]ATP on lysates of NIH 3T3 cells. Kinase assays of immunoprecipitates produced with an antiserum directed against the ARK extracellular domain resulted in the appearance of a phosphorylated band of approximately 120 kDa that was not present in the immunoprecipitates obtained with preimmune sera (data not shown).

We then analyzed the level of ARK phosphorylation in NIH 3T3 cells. Cells were starved for 48 h with 0.2% serum, and the cell lysate was immunoprecipitated with anti-ARK antiserum. Immunoprecipitates were then subjected to Western blotting (immunoblotting) analysis using either anti-ARK or antiphosphotyrosine antibodies. As shown in Fig. 1, the molecule of 120 kDa recognized by the anti-ARK antiserum (lane 1) appears to be phosphorylated (lane 2), indicating that the ARK receptor is present in an activated form. Similar results were obtained for NIH 3T3 cells grown in 10% serum or for transfected CHO cells (not shown).

These results indicate that ARK expression in NIH 3T3 or transfected CHO cells is accompanied by receptor phosphorylation, possibly resulting from the presence of the ARK ligand on the cell surface or culture medium of CHO and NIH 3T3 cells. A variety of CAMs exhibit homophilic binding. We therefore investigated the possibility that ARK activation resulted from binding to ARK molecules present on the surface of cells expressing this receptor.

Expression of ARK in *Drosophila* S2 cells induces aggregation. To study whether the ARK protein was capable of homophilic binding, we expressed ARK in *Drosophila* S2 cells and determined whether that resulted in increased aggregation. The S2 cell line has been used in many cases for this purpose because of its low level of spontaneous aggregation (35), and we also considered it unlikely that insect cells spontaneously expressed a conserved ligand for the ARK protein. Two constructs expressing the mouse ARK protein under the control of inducible promoters were transfected into S2 cells. The full-

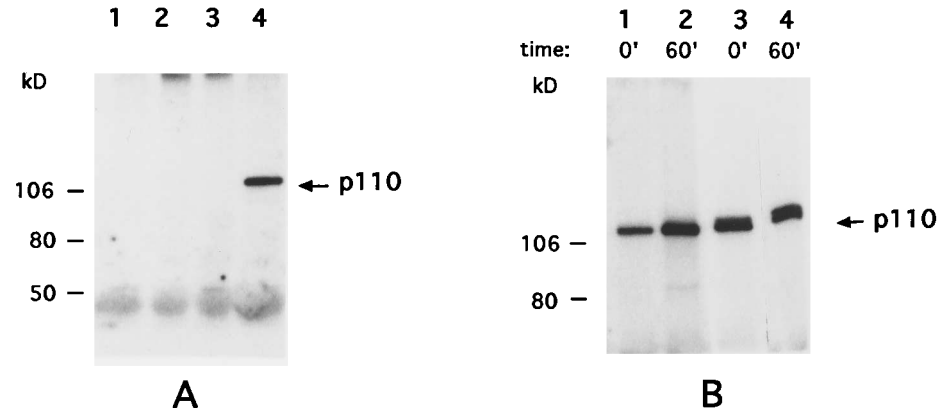

FIG. 5. Expression and phosphorylation of the ARK protein in transfected S2 cells. S2 cells stably transfected with the *ark* cDNA in both sense and antisense orientations were induced for 30 min at 37°C. After recovery at room temperature, the cells were assayed for aggregation and the level of expression of the ARK protein was analyzed by Western blotting. (A) Cells were lysed in 1% Triton buffer and incubated with an antiserum against the intracellular domain of the ARK protein. Immunoprecipitates were separated by SDS-PAGE (6% gel) and immunoblotted with anti-ARK receptor antiserum. Lanes: 1, antisense, uninduced; 2, antisense, induced; 3, sense, uninduced; 4, sense, induced. The migration of the ARK protein (p110) is indicated on the right. Molecular weight markers are indicated on the left. (B) Lysates from ARK-expressing cells before (lane 1 and 3) or after (lanes 2 and 4) aggregation for 60 min (60′) were incubated with anti ARK receptor antiserum. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-ARK antiserum (lanes 3 and 4) or with antiphosphotyrosine antibodies (lanes 1 and 2). The migration of the ARK protein (p110) is indicated on the right. Numbers on the left indicate molecular mass markers.

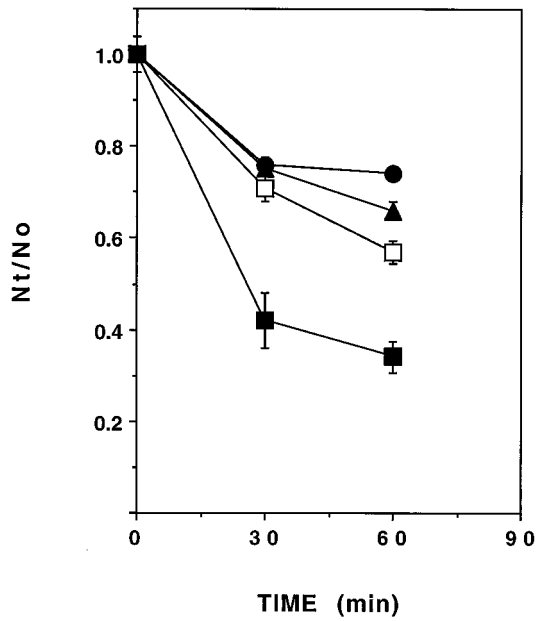

FIG. 6. Kinetics of aggregation by S2 cells expressing the truncated ARK receptor. S2 cells stably transfected with the truncated ARK-TKΔ receptor, in which the entire tyrosine kinase domain was deleted, were assayed for aggregation as described in Materials and Methods. Quantitation of aggregate formation was monitored with a Coulter Counter. Since aggregation leads to a decrease of the total number of particles in suspension, aggregation was quantitated by dividing the number of particles in suspension measured at time *t* (Nt) by the number of particles detected at time zero (No). ▲, untransfected S2 cells, induced; ●, S2 cells transfected with the cDNA encoding the truncated ARK-TKΔ receptor, uninduced; ■, induced ARK-TKΔ-transfected S2 cells in which aggregation was performed in the presence of 100 μg of anti-ARK Fab′ fragments (□) or preimmune Fab′ (■) per ml. The standard error at each time point is indicated by a vertical bar ($n = 3$).

length *ark* cDNA was cloned into pCaSpeR vector under the control of *hsp70* promoter (39). As a control, a chimeric receptor *bek-ark* cDNA, in which the portion of *ark* cDNA coding for the extracellular domain of the protein was substituted by the extracellular portion of FGFR-2 (BEK) (24), was cloned into the pRmHa-3 vector under the control of the metallothionein promoter (5). S2 cells were transfected with these plasmids as described in Materials and Methods, and stably transfected cell lines were isolated. Expression of the ARK protein, which in this case migrates like a 110-kDa protein, probably because of lower levels of glycosylation, was easily detected after induction (see Fig. 5A). FACS analysis performed on live transfected S2 cells by using an antibody which recognizes ARK extracellular domain showed that the ARK molecule was correctly express on the cell surface (data not shown).

We induced S2 cells stably transfected with the full-length *ark* cDNA in the sense or antisense orientation and S2 cells stably transfected with *bek-ark* cDNA and assayed them for aggregation as described in Materials and Methods. Formation of aggregates was visualized with a phase-contrast microscope. We observed a large number of aggregates consisting of 10 to 25 cells only in the induced S2 cells expressing ARK (Fig. 2A). Addition of anti-ARK (extracellular) Fab′ fragments, at the concentration of 100 μg/ml, strongly inhibited the formation of large aggregates (Fig. 2B). No aggregates were observed in induced S2 cells transfected with the vector containing the *ark* cDNA in the antisense orientation (Fig. 2D) or in induced S2 cells transfected with the *bek-ark* cDNA (Fig. 2C). It appears, therefore, that expression of the ARK protein in S2 cells results in increased aggregation.

ARK mediates homophilic aggregation in S2 cells. The results presented above indicated either that ARK expression induced S2 cell aggregation by homophilic binding or that aggregation resulted from the fact that the S2 cells used for

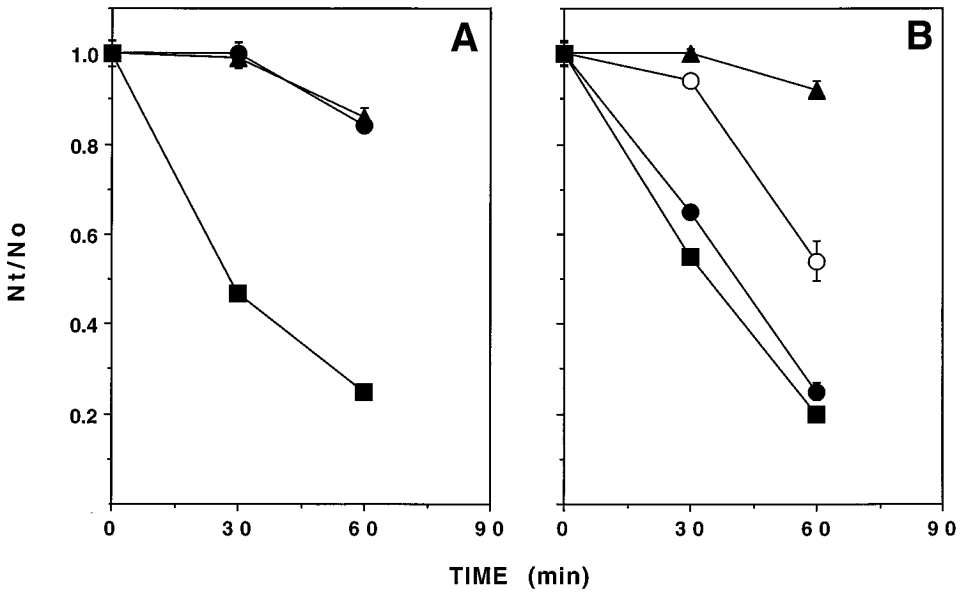

FIG. 7. Aggregation induced by the ARK protein in mammalian cells. Cells were collected as described in Materials and Methods and allowed to aggregate at 90 rpm at 37°C in S-MEM containing 10% dialyzed calf serum. Aliquots were removed at the indicated times, and the total number of particles was counted with a Coulter Counter. Aggregation was measured by the ratio of the number of particles detected at time *t* (nt) to the number of particles at time zero (No) as described in the legend to Fig. 6. The aggregation assay was performed with transfected CHO cells. ■, cells overexpressing the ARK receptor; ●, cells expressing a chimeric receptor, BEK-ARK, in which the extracellular portion of the ARK receptor was substituted with the extracellular domain of FGFR-2 (BEK); ▲, untransfected CHO-DG44 cells. (B) NIH 3T3 cells overexpressing the truncated ARK-TKΔ receptor (■); NIH 3T3 cells expressing ARK-TKΔ and incubated with 100 μg of Fab′ anti-ARK antibodies (○) or Fab′ from preimmune serum (●) per ml; NIH 3T3 cells transfected with *ark* cDNA in the antisense orientation (▲). The standard error at each time point is indicated by a vertical bar ($n = 3$).

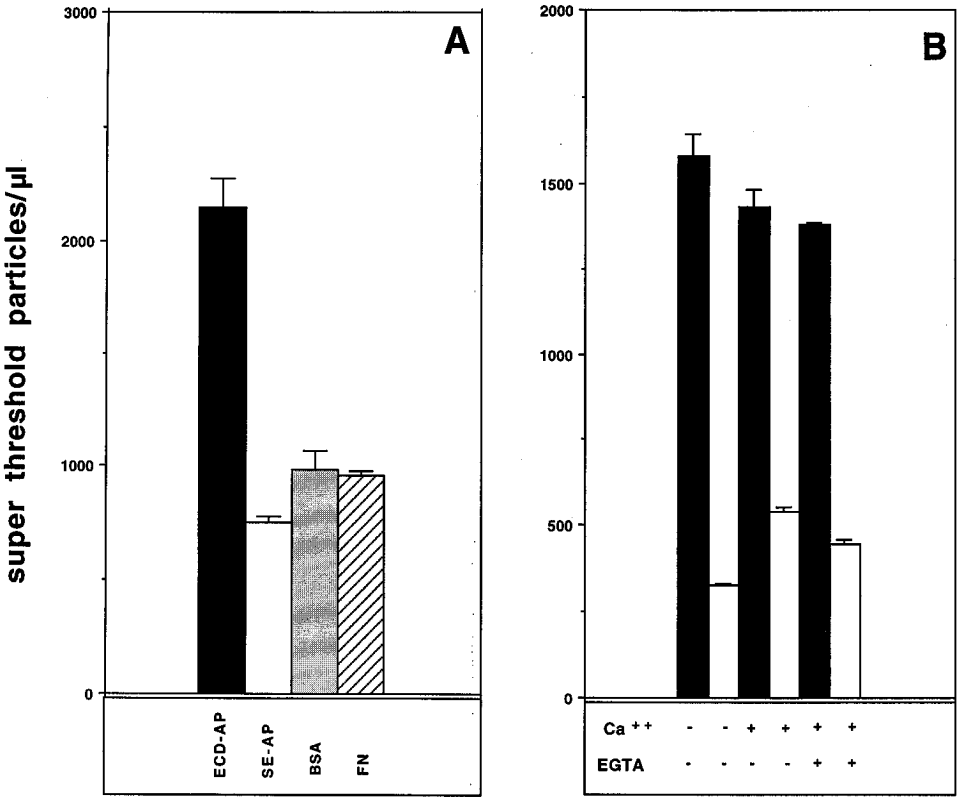

FIG. 8. Aggregation of fluorescent beads (Covaspheres) coated with the purified extracellular domain of ARK. (A) The beads were covalently coated with different proteins and allowed to aggregate at room temperature in PBS. Aggregation was monitored after 1 h with a Coulter Counter as described in Materials and Methods. Standard errors are indicated by the vertical bars. (B) Aggregation was performed with ECD-AP (■)- or SE-AP (□)-coated Covaspheres in the presence or absence of $CaCl_2$ (1 mM) or EGTA (1 mM).

these experiments expressed a molecule that bound to ARK heterophilically. To distinguish between these possibilities, we stained control or ARK-expressing S2 cells with the lipophilic dye DiI and used them to perform mixing experiments with unlabeled cells of either type. The presence of cells of either population in the aggregates was monitored by fluorescence microscopy. When equal numbers of labeled and unlabeled ARK-expressing cells were allowed to aggregate, we observed the formation of mixed aggregates consisting of stained and unstained cells (Fig. 3B). However, mixing of unlabeled ARK-expressing cells with labeled negative cells led to the formation of aggregates consisting only of unlabeled cells (Fig. 3C), and vice versa (not shown), indicating that aggregation is mediated by homophilic binding. No aggregates were observed when populations of labeled and unlabeled control cells were tested (Fig. 3A).

A more quantitative analysis of this experiment is shown in Fig. 4, which confirms the observation that when DiI-stained, ARK-expressing cells are mixed with unstained untransfected cells, the aggregates are composed mainly of ARK-expressing cells. Mixed aggregation experiments using cells expressing the BEK-ARK chimera showed that these cells did not form aggregates with ARK-expressing cells (data not shown).

Cell aggregation increases tyrosine phosphorylation of ARK. To determine whether homophilic binding mediated by ARK leads to activation of the receptor itself, we examined its level of phosphorylation during the aggregation process. Induced ARK-transfected S2 cells were allowed to aggregate under standard conditions. Cells extracts were incubated with anti-ARK antiserum, and the immunoprecipitates were subjected to Western blotting analysis using either anti-ARK or antiphosphotyrosine antibodies. As shown in Fig. 5B, a basal level of phosphorylation of ARK is observed in cells that were not allowed to aggregate. Tyrosine phosphorylation, however, showed a small but reproducible increase during aggregation, suggesting that homophilic binding led to the activation of the ARK receptor. PhosphorImager analysis showed that the increase in phosphorylation was about threefold. An additional phosphorylated protein of about 85 kDa is also detectable in the immunoprecipitates from aggregated cells (Fig. 5B, lane 2). This protein may represent an ARK substrate which interacts with the activated ARK receptor.

ARK-mediated aggregation does not require its cytoplasmic domain. To study whether adhesion required tyrosine kinase activity, we constructed a cDNA in which the sequences coding for the intracellular domain of ARK were deleted downstream of the transmembrane region as described in Materials and Methods. The resulting cDNA molecule encodes a 522-amino-acid polypeptide which includes an intact ARK extracellular domain, the transmembrane region, and the first 56 amino acids of its cytoplasmic portion but lacks the entire tyrosine kinase domain. The cDNA expressing this truncated receptor (ARK-TKΔ) was cloned in a *Drosophila*-inducible expression vector, and S2 cells stably transfected with this construct were assayed for aggregation. As shown in Fig. 6, after 30 min, ARK-TKΔ-expressing cells also showed a high level of aggregation. In addition, aggregation was inhibited by anti-ARK extracellular domain Fab′ fragments. Thus, ARK-TKΔ-ex-

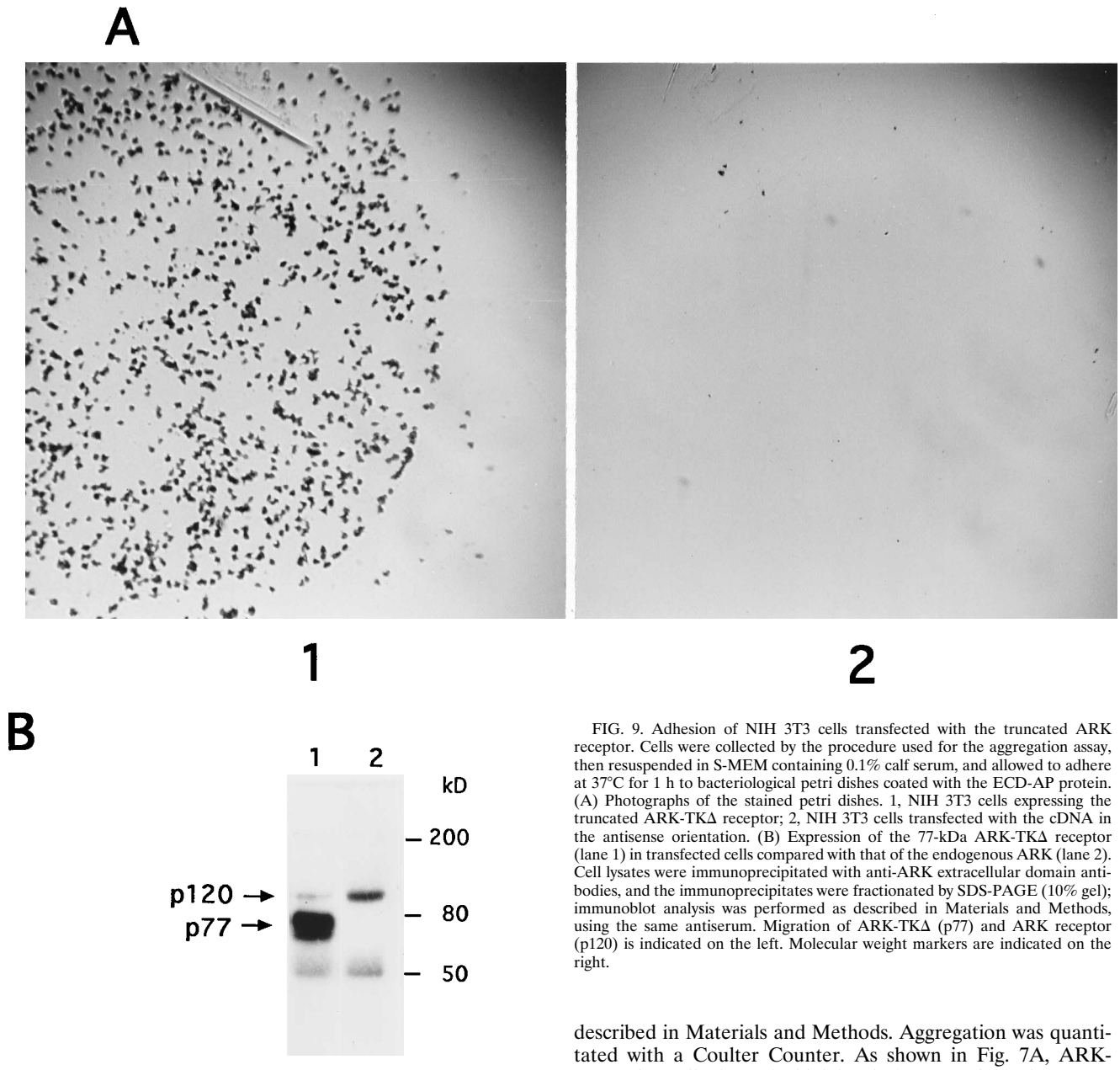

FIG. 9. Adhesion of NIH 3T3 cells transfected with the truncated ARK receptor. Cells were collected by the procedure used for the aggregation assay, then resuspended in S-MEM containing 0.1% calf serum, and allowed to adhere at 37°C for 1 h to bacteriological petri dishes coated with the ECD-AP protein. (A) Photographs of the stained petri dishes. 1, NIH 3T3 cells expressing the truncated ARK-TKΔ receptor; 2, NIH 3T3 cells transfected with the cDNA in the antisense orientation. (B) Expression of the 77-kDa ARK-TKΔ receptor (lane 1) in transfected cells compared with that of the endogenous ARK (lane 2). Cell lysates were immunoprecipitated with anti-ARK extracellular domain antibodies, and the immunoprecipitates were fractionated by SDS-PAGE (10% gel); immunoblot analysis was performed as described in Materials and Methods, using the same antiserum. Migration of ARK-TKΔ (p77) and ARK receptor (p120) is indicated on the left. Molecular weight markers are indicated on the right.

pressing cells are as capable of aggregation as cells expressing the wild-type ARK receptor, indicating that the ARK tyrosine kinase domain is not required for aggregation.

High levels of expression of ARK in CHO and 3T3 cells also induce aggregation. The results obtained with S2 cells showed that ARK could mediate cell aggregation through homophilic binding. We wished to determine whether ARK also induced or increased aggregation in mammalian NIH 3T3 or CHO cells. Since these cells already express endogenous ARK, we transfected them to express high levels of ARK and measured their ability to undergo increased aggregation. We first tested dihydrofolate reductase-negative CHO cells transfected with either full-length *ark* cDNA or the chimeric *bek-ark* construct in the p91023(B) vector. Stable transfectants expressing high ARK levels were selected and subjected to aggregation as described in Materials and Methods. Aggregation was quantitated with a Coulter Counter. As shown in Fig. 7A, ARK-expressing cells showed a high level of aggregation, whereas no significant aggregation was detected in cells expressing the chimera BEK-ARK or in the untransfected parental DG44 cells.

We were not able to obtain NIH 3T3 cells which overexpressed full-length ARK to a significant degree, perhaps because ARK overexpression is detrimental to cell growth. We therefore used cells which had been transfected with a mammalian vector expressing the truncated (ARK-TKΔ) molecule, since we had already shown that this truncated receptor was capable of promoting aggregation in S2 cells. Stable clones expressing high levels of truncated ARK were selected, and aggregation assays were performed as with CHO cells. Figure 7B shows that such cells are capable of increased aggregation with respect to the control; furthermore, aggregation was significantly inhibited by anti-ARK Fab′ fragments. Taken together, these results show that ARK is capable of inducing cell aggregation also in a mammalian cell system and that

TABLE 1. Adhesion of cells expressing ARK to surfaces coated with ECD-AP[a]

Protein	No. (%) of adherent cells/coated area			
	ARK-transfected S2 cells		NIH 3T3 cells	
	Uninduced	Induced	Control[b]	ARK-TKΔ transfected
ECD-AP	0[c]	2,350 ± 100 (17)	0	458 ± 9 (21)
ECD-AP[d]	ND[e]	ND	0	109 ± 8 (5)
SE-AP	0	0	0	0
BSA	0	0	0	0
FN	13,600 ± 374 (100)	13,866 ± 618 (100)	3,000 ± 489 (100)	2,130 ± 126 (100)

[a] Adhesion assays were performed in bacteriological petri dishes in which small areas were coated with purified ARK ECD-AP or control proteins. The dishes were then incubated with different populations of cells as described in Materials and Methods. The number of cells bound is represented as the mean of three experiments. The numbers indicate total cells bound to each area ± standard errors. In parentheses are the percentages of cells bound relative to the number of cells bound to FN.
[b] NIH 3T3 cells transfected with the *ark-tk*Δ cDNA in the antisense orientation.
[c] 0, too few to count.
[d] Fab′ fragment raised against the ARK extracellular domain (100 μg/ml) was added to the cells during the assay.
[e] ND, not done.

aggregation requires only the extracellular domain of the receptor.

The extracellular domain of ARK can mediate homotypic aggregation in vitro in a Ca^{2+}-independent manner. We next studied whether the extracellular domain of ARK was able to mediate aggregation in vitro. To perform these experiments, the ARK extracellular domain was produced in NIH 3T3 cells as a secreted protein fused at its C terminus with the placental alkaline phosphatase gene (ECD-AP). Conditioned medium of a stable NIH 3T3 producing clone was subjected to affinity chromatography using an anti-alkaline phosphatase monoclonal antibody (11). The partially purified ECD-AP protein was covalently coupled to synthetic fluorescent beads (Covaspheres) and tested for its ability to promote aggregation. As shown in Fig. 8, Covaspheres coated with ECD-AP protein rapidly aggregated. A lower number of aggregates was observed with Covaspheres coated with SE-AP, FN, or BSA (Fig. 8A). Addition of Ca^{2+} or EGTA during the assay did not influence the number of aggregates, indicating that binding occurs in a Ca^{2+}-independent manner (Fig. 8B).

The ECD of ARK can function as a specific substrate for adhesion by ARK-expressing cells. To further confirm that the increased aggregation promoted by ARK expression was the result of homophilic binding, adhesion assays were performed in bacteriological petri dishes in which small areas were coated with purified ARK ECD-AP or control proteins. The dishes were then incubated with different populations of cells for 1 h. Cells attached were stained and counted under a microscope (Fig. 9). As shown in Table 1, when S2 cells were used, only the induced ARK-expressing cells bound to the coated ECD-AP protein. No binding was observed with uninduced ARK-expressing cells. In the case of mammalian cell lines, we observed adhesion to the ECD-AP protein by NIH 3T3 cells overexpressing the truncated ARK receptor. Control NIH 3T3 cells, which express endogenous ARK, did not bind to ECD-AP-coated plates appreciably (Table 1 and Fig. 9). We could not detect adhesion of NIH 3T3 cells overexpressing ARK-TKΔ to a plate coated with the extracellular domain of R-PTP-κ, which has also been shown (31) to be capable of homophilic binding (data not shown). These results indicate that the ECD-AP protein can promote adhesion of cells that express ARK but that under the conditions of our experiments, the expression of a high number of receptors is necessary to mediate a significant degree of adhesion to a substrate coated with purified ARK protein.

In situ analysis of ARK mRNA in the adult mouse brain. Studies of in situ hybridization in the developing mouse embryo had detected high expression of UFO (i.e., ARK) RNA in many organ stromas (9). On the other hand, our previous RNA blot analysis on mouse adult tissues revealed high levels of ARK mRNA in the brain (30). To determine which structures in the adult mouse brain express ARK mRNA, we performed in situ hybridization on mouse brain sections, using as a probe the 466-bp fragment of the extracellular portion of the receptor. As shown in Fig. 10, a high level of expression of ARK mRNA was found in the hippocampus and cerebellum. Figure 10A shows staining of the pyramidal cell layer of the hippocampus as well of the granular layer of the dentate gyrus; Fig. 10B shows expression of ARK mRNA in the cerebellar cortex, and high magnification (Fig. 10C) of the cerebellar cortex shows that ARK is expressed predominantly in Purkinje cells. It is interesting that this pattern of expression is similar to but distinct from that of Tyro 3, another member of the ARK receptor family (23).

DISCUSSION

The isolation of ARK, a putative murine RTK, as well as of its human homolog AXL or UFO, showed for the first time the existence of a transmembrane molecule which combined features of a typical RTK with those of CAMs. This finding raised the obvious question of whether this molecule could be involved in transmitting signals originating from cell-to-cell contact or from the interaction with components of the extracellular matrix. It is known, for example, that integrins do not possess tyrosine kinase activity but that their signal transduction pathways involve proteins which are capable of phosphorylating or being phosphorylated at tyrosine residues (21). ARK could therefore be a CAM which has evolved toward a direct and specific mechanism of tyrosine phosphorylation. We therefore undertook the characterization of the binding properties and enzymatic activity of ARK in the hope of obtaining clues as to the protein's biological function.

The results presented in this report show that ARK is capable of homophilic binding and that ARK-ARK interaction can lead to a degree of receptor activation. Expression of ARK in insect cells leads to significantly increased cell aggregation, and we demonstrated that this resulted from a specific interaction between ARK-expressing cells. Thus, ARK is capable of homophilic binding, and furthermore, we found that this binding does not require the presence of the intracellular, enzymatic moiety of the receptor. We obviously cannot exclude at this point the possibility that ARK can also interact with an unknown heterophilic ligand. In this respect, it is worth mentioning that when we performed mixed aggregation experiments

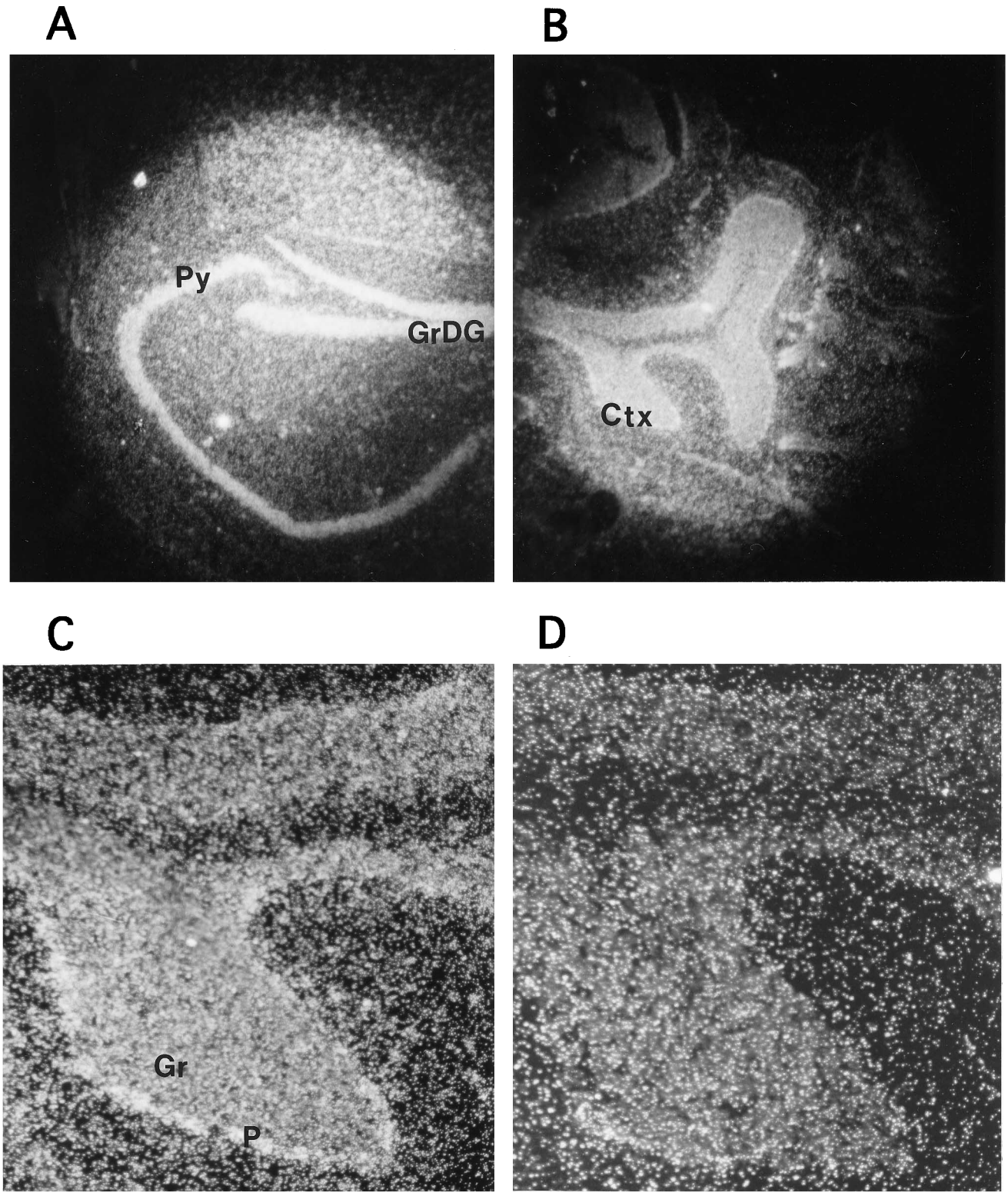

FIG. 10. In situ hybridization analysis of ARK mRNA in the brain. Localization of ARK mRNA in sagittal sections of adult mouse brains was determined by using a ^{35}S-labeled fragment of 466 bp from the extracellular domain of the receptor. Intense hybridization is detected in the pyramidal cell layer of the hippocampus (Py) and granular cell layer of the dentate gyrus (GrDG) (A) and in the cerebellar cortex (Ctx) (B). Higher magnification of the cerebellar cortex shows ARK mRNA expression preferentially in Purkinje cells (P) (C). No specific hybridization was apparent in the cerebellar cortex when the corresponding sense RNA probe was used (D).

with NIH 3T3 or CHO cells, using transfected and parental cells stained with DiI, many of the aggregates contained both transfected and untransfected cells. In S2 cells, in contrast, aggregates consisted only of ARK-expressing cells. It is likely that this finding reflects the fact that unlike S2 cells, untransfected NIH 3T3 and CHO cells express a significant level of endogenous ARK protein, but it is also possible that an unidentified heterophilic ligand is expressed on their surface.

In insect cells, aggregation leads to an increase in receptor autophosphorylation. Although the increase is not very high (about threefold), probably because of the relatively high degree of constitutive phosphorylation, the appearance of an additional phosphorylated protein in the immunoblots of aggregated cells supports the hypothesis that ARK homophilic binding leads to activation of the receptor. It is also possible that the ARK tyrosine residues phosphorylated in aggregated cells are not the same residues phosphorylated prior to aggregation. The new band could represent an ARK substrate which is associated with the activated ARK protein and thus is specifically brought down during immunoprecipitation. Its nature is under investigation.

In NIH 3T3 or CHO cells, on the other hand, we showed that under the experimental conditions used so far, ARK appears to be constitutively phosphorylated; this is also the case with insect cells, although an increase can be seen after aggregation. Constitutive phosphorylation of ARK in NIH 3T3 cells, presumably due to receptor activation, could be attributable to several mechanisms: (i) the presence of an unidentified heterophilic ligand; (ii) homophilic binding and dimerization between receptors expressed on the same cell; and (iii) production of a soluble form of ARK, which could be present in the medium or associated with the extracellular matrix. Preliminary results indicate that ARK can be cleaved into a soluble form which is detected in the medium of NIH 3T3 cells. Whether this form is responsible for the observed ARK activation remains to be determined. As mentioned above, it is also possible that the apparently constitutive phosphorylation of ARK in NIH 3T3 cells derives not from receptor activation but from the phosphorylation of tyrosine residues that are different from those phosphorylated after activation. This matter is under investigation.

In S2 and NIH 3T3 cells, we also found that a high degree of expression of the ARK extracellular domain can promote adhesion to substrates coated with a recombinant ARK protein. While this observation strengthens the belief that ARK is capable of homophilic binding, it raises the question of why the expression of endogenous ARK in 3T3 cells is not sufficient to promote adhesion. Although several explanations are possible, we consider it likely that the recombinant ARK protein used as an adhesion substrate in these experiments does not have an affinity for the transmembrane ARK high enough to allow the detection of significant adhesion unless ARK is expressed in very large amounts on the adhering cells.

The only tyrosine kinase receptor which has been shown to be involved in homophilic binding is D-TRK, a neuronal CAM which belongs to the family of neurotrophins receptors (29). We do not know whether ARK plays a specific role in the nervous system, although its high level of expression in the brain suggests that it may do so. Two other molecules putatively involved in signal transduction, the receptor phosphatases R-PTP-μ and R-PTP-κ, are also capable of homophilic binding (4, 31). It is worth noting that the ARK extracellular domain bears considerable resemblance to those of these two phosphatases in its overall structure.

Recently, the isolation of a number of cDNAs encoding proteins homologous to but distinct from ARK has been reported (12, 23, 25, 28). Although it is not yet clear how many of these cDNAs represent distinct genes, these findings point to the emergence of a new family of RTKs, with ARK (AXL, UFO) being its prototype. Expression of these new *ark*-related genes appears to be predominant in the central nervous system. On the other hand, Northern blot and in situ hybridization experiments reveal that ARK expression is rather widespread but not ubiquitous (30). In tissue culture, ARK is expressed in a variety of cell lines of fibroblastic or epithelial cell origin but not in hematopoietic cell lines or in primary hematopoietic tissues (27). In vivo, ARK RNA begins to be expressed in the mouse embryo at approximately day 12.5 postcoitum and is then expressed in a variety of solid tissues, particularly in structures of mesodermal origins which constitute the stroma of several organs, including connective tissue (9). As shown in this report, ARK is also expressed at very high levels in the brain, specifically in the hippocampus and the cerebellum. Strikingly, the expression of Tyro 3, a new member of the ARK family, is also very high in the hippocampus and cerebellum (23) but is confined to structures distinct from those in which ARK is highly expressed. This pattern of expression is consistent with the hypothesis that these molecules may play a role at cellular junctions and generate growth-regulatory or differentiation signals originating from cell-to-cell contact.

In conclusion, that data presented in this report indicate that ARK is an RTK which is capable of mediating homotypic binding and that such binding result in activation of tyrosine kinase activity. The physiological function of ARK and the type of signals that ARK homotypic interaction generates are currently under investigation.

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