Chimeric transmembrane receptors of eucaryotic origin have been constructed in which extracellular, transmembrane, and intracellular domains are combined in order to study receptor function. The resulting hybrids have often exhibited transmembrane signaling. For instance, insulin binding to an extracellular insulin receptor domain was shown to activate an intracellular kinase domain derived from the epidermal growth factor receptor (1). A functional hybrid was constructed between the platelet-derived growth factor receptor and the fibroblast growth factor receptor (2). Chimeric procaryotic receptors between domains from the Escherichia coli aspartate receptor (AR) and EnvZ receptor have also been shown to transduce signals (3). In addition, two different hybrids derived from the procaryotic E. coli AR extracellular domain and the human insulin receptor kinase domain have been expressed and studied in E. coli (4, 5), one of which was regulated by aspartate.

Despite a lack of sequence similarity or shared biochemical activities, the IR and AR have some congruence. The AR is a homodimer of 60 kDa subunits with noncovalent contacts between the extracellular and possibly the transmembrane domains (6, 9). Each subunit has two transmembrane domains that flank the extracellular aspartate binding domain. Two identical aspartate binding sites have been identified in the dimer by equilibrium binding studies and x-ray diffraction imaging that are known to interact leading to negatively cooperative ligand binding (7, 8). The IR configuration is analogous in that two αβ transmembrane subunits join as a “dimer” (αβ)2, but they are covalently linked by a disulfide as well as noncovalent bonds (9). Each αβ molecule has an insulin binding site, and the αβ2 receptor exhibits negatively cooperative insulin binding (10, 11).

Both receptors transmit signals across the membrane by conformational changes because receptor clustering mechanisms have been ruled out (6, 9). Small conformational changes that may be involved in signaling have been observed in AR crystal structures and nuclear magnetic resonance studies (7, 12). No similar structural data are available for the IR, but studies of its signaling mechanism have indicated that binding of an insulin molecule by one αβ subunit has the initial effect of inducing autophosphorylation of the other subunit (13). Inter-subunit autophosphorylation precedes activation of the exogenous kinase activity of the IR (14).

The aspartate/insulin receptor chimera (AIR) when expressed in procaryotes responds to aspartate binding with stimulated kinase activity (5). It was of interest to examine how a eucaryotic system would handle this hybrid containing procaryotic ligand-binding and transmembrane domains and whether the resulting receptor could function. We have therefore expressed AIR in mammalian cells and studied its enzymatic activity, subcellular location, and interactions with cellular mitogenic pathways.

MATERIALS AND METHODS

Cells and Antisera—Murine 3T3 fibroblasts (15) were obtained from the laboratory of Richard Mulligan and were cultured under standard conditions in Dulbecco’s modified Eagle’s medium containing 10% calf serum and antibiotics. Rabbit antiserum 9207 was generated by immunization of a female New Zealand White rabbit with purified AR expressed in E. coli. AR-specific IgG was isolated by affinity purification on a column of recombinant AR cross-linked to Affi-gel 10 (Bio-Rad) essentially as described (16).

Expression of AIR—Plasmid pLJAIR was constructed by subcloning the BamHI-StuI DNA fragment from pLAIR (5) containing the AIR gene into retroviral expression vector pLJ (17) that had been cut with the same two enzymes. CeCl-purified DNA was used in calcium phosphate transfections of the fibroblast line 3T3. Colonies were isolated after selection of transfected cells in 500 μg/ml G418, and clone CA3 was chosen for further study on the basis of immunoblot screening of cell lysates with anti-AR serum 9207. AIR expression was found to be maintained for more than 20 passages under G418 selection, and experiments were carried out on AIR transfectants that had been passaged 3–15 times.

Cell Lysis and Labeling—Particulate and soluble fractions were pre-
pared for biochemical studies at 4 °C by lysing cells (5–10 × 10^6) in a volume of 1 ml using a Dounce homogenizer with tight fitting pestle (25 strokes) in lysis buffer (20 mM HEPES, 1 mM EDTA, 40 μg/ml phenylmethylsulfonyl fluoride, pH 7.3) and centrifugation at approximately 200,000 × g for 10 min. Membrane pellets were extracted in lysis buffer containing 0.5% Triton X-100 for 15 min and centrifuged as above to isolate solubilized proteins. Samples were normalized for equivalent containing 0.5% Triton X-100 for 15 min and centrifuged as above to isolate solubilized proteins. Samples were normalized for equivalent protein concentrations with the BCA protein assay (Pierce).

Metabolic labeling of cells with [32P]orthophosphate was performed with nearly confluent 100-mm dishes of cells that had been cultured in 0.5% serum for 22 h, washed in phosphate- and serum-free medium, and labeled with 0.1 mCi/ml [32P]orthophosphate in the same medium (0.5% calf serum) for 30 h and then stimulated with 10% calf medium (0.5% calf serum) for 18 h or left unstimulated. 1 μCi/ml [3H]thymidine was added for 1 h before harvesting, cells were washed in PBS, and measurement of acid-insoluble counts was as described (18).

**Receptor Analyses—** Immunoprecipitations were performed in 1 ml for 1.5 h at 4 °C in lysis buffer plus Triton X-100 (for kinase assays) or RIP A buffer plus 0.1 mM sodium vanadate, and solubilized proteins were isolated by centrifugation at 200,000 × g for 12 min.

Thymidine incorporation was measured in six-well plates with subconfluent cells that had been starved in Dulbecco's modified Eagle's medium (0.5% calf serum) for 30 h and then stimulated with 10% calf serum for 18 h or left unstimulated. 1 μCi/ml [3H]thymidine was added for 1 h before harvesting, cells were washed in PBS, and measurement of acid-insoluble counts was as described (18).

**Immunofluorescence—** Cells were grown on coverslips and fixed, permeabilized, and stained under conditions essentially as described (20). Coverslips were washed twice with PBS and fixed with 2% paraformaldehyde in PBS, quenched, and then permeabilized in 0.3% Triton X-100/PBS. Nonspecific binding sites were blocked by sequential incubations in PBS solutions containing 2% normal goat serum, 2% egg albumin, and 5% nonfat dry milk. Affinity-purified anti-AR antibodies (approximately 3–10 μg that had been prebound to protein A beads) and followed by four washes in the same buffer. Immunoprecipitates corresponding to approximately 10^6 cells were electrophoresed on 10% denaturing polyacrylamide gels, and autoradiography was performed.

Fifty-microliter kinase reactions were carried out for 15 min at room temperature in 20 mM HEPES, 10 mM MgCl₂, 10 mM MnCl₂, 40 μg/ml phenylmethylsulfonyl fluoride, 20 μM [γ-32P]ATP (5 μCi), pH 7.3, with or without 0.5 mM histone H2B (Boehringer Mannheim). Densitometry of SDS-polyacrylamide gel autoradiograms was performed with a digital imaging system (Alpha Innotech IS-1000). Phosphoamino acid analysis of proteins in gel slices was performed by electrophoresis in one dimension only at pH 3.5 essentially as described (5). All autoradiography was with Kodak X-AR film exposed at E.I. 400-1200. Autoradiography was performed. Fifty-microliter kinase reactions were carried out for 15 min at room temperature in 20 mM HEPES, 10 mM MgCl₂, 10 mM MnCl₂, 40 μg/ml phenylmethylsulfonyl fluoride, 20 μM [γ-32P]ATP (5 μCi), pH 7.3, with or without 0.5 mM histone H2B (Boehringer Mannheim). Densitometry of SDS-polyacrylamide gel autoradiograms was performed with a digital imaging system (Alpha Innotech IS-1000). Phosphoamino acid analysis of proteins in gel slices was performed by electrophoresis in one dimension only at pH 3.5 essentially as described (5). All autoradiography was with Kodak X-AR film exposed at E.I. 400-1200. Autoradiography was performed.

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ability of the IR to autophosphorylate in vitro is conserved in AIR, isolated CA3 cellular membranes were incubated with radiolabeled ATP and then immunoprecipitated with anti-AR antibody. Fig. 2 shows that whereas no phosphorylated bands were recognized in 3T3 parental cell membranes (lane 1), a single radiolabeled band of 70 kDa is found in CA3 membranes (lane 2). No band was observed in analyses of cytoplasmic proteins (not shown). The slight increase in apparent molecular mass of the protein in the phosphorylated band relative to the band in Fig. 1A is likely to be attributable to a different phosphorylation state. Phosphatase experiments demonstrated that AIR autophosphorylation levels regulate the exogenous kinase activity of the receptor in immunoprecipitates. Treatment of immunoprecipitates with 10 units acid phosphatase for 5 min followed by extensive washing reduced histone kinase activity by 50% (data not shown).

AIR was found to be phosphorylated in cells metabolically labeled with $^{[32P]}$orthophosphate. Fig. 3A shows a phosphorylated doublet in immunoprecipitates from solubilized CA3 cells (lane 4) that is not recognized by preimmune serum (lane 2) and is absent in untransfected parental cells (lanes 1 and 3). Evidence that phosphotyrosine was present on AIR within the cell is presented in the phosphotyrosine Western blot in Fig. 3B. Proteins from the particulate fraction of CA3 cells had a predominant band of the appropriate size (lane 1) that was not present in parental cells (lane 2).

Subcellular Location of AIR—The presence of an active full-length AIR kinase in the particulate fraction of CA3 cells suggested that the receptor was folded to the active conformation and was inserted into cellular membranes. Double-label immunofluorescence revealed that significant amounts of AIR could be detected only in the perinuclear region corresponding to the endoplasmic reticulum and Golgi membranes and not in the plasma membrane by the aspartate receptor antibody (Fig. 4A). This specific subcellular location is suggested because double-label immunofluorescence of CA3 cells with AR Ab and concanavalin A-fluorescein isothiocyanate conjugate showed very similar staining patterns (Fig. 4A and B). Confirmation that the AR antibody was indeed detecting AIR is provided by the absence of staining of CA3 cells when the antibody had been preincubated with excess AR protein (Fig. 4C). Likewise, no staining was observed with AR antibody on parental cells (Fig. 4D) or with preimmune antibody on CA3 cells (not shown). Because the N-terminal region containing the transmembrane domains has procaryotic sequences, the failure of transport to the exterior membrane is understandable.

Induction of DNA Synthesis by AIR—To see whether the chimeric receptor exhibited some of the insulin receptor’s regulatory properties, we measured thymidine uptake in serum-deprived cells. AIR was found to induce thymidine uptake as does the native insulin receptor kinase. Subconfluent monolayers of CA3 and parental cells that had been serum-deprived for 30 h and then stimulated with calf serum or left untreated were labeled with $[^3]H$thymidine, and the incorporated radiolabel was measured. Fig. 5 shows that serum-deprived 3T3 parental cells lacking AIR incorporated minimal amounts of thymidine but upon serum stimulation took up large amounts of thymidine. CA3 cells, on the other hand, took up thymidine with or without stimulation, indicating that they were mitogenically activated independently of external growth factors.

**DISCUSSION**

We report here that the hybrid AIR can be expressed in mouse 3T3 cells and enters the endoplasmic reticulum where it is apparently retained. This intracellular protein is an active tyrosine kinase that is capable of stimulating DNA synthesis under serum deprivation conditions.
Insulin receptors lacking most of the cytoplasm of receptors in general (22, 23) and the IR in particular ticulum retention are consistent with prevailing views of traf-
the expression of a kinetically functional receptor but do affect and the absence of indicating that the result after treatment of the native IR with dithiothreitol in-
rylate on tyrosine datasupportamodelinwhichtheAIRsubunitstransphos-
dimerize via noncovalent intersubunit interactions (28). Our
binding and transmembrane domains have been shown to
uration (10). We proposethatAIRisdimericbecauseitsligand
exogenous substrates. Our in vitro phosphatase experiments con-
firm that the high AIR exogenous kinase activity we see is regulated by autophosphorylation as in the case of the IR.

This report shows that AIR is permanently “turned on” in vitro, and we propose that as with the insulin-bound IR, transphosphorylation takes place and cellular substrates are phosphorylated that transduce signals to the metabolic ma-
inery. The mechanism of activation may be independent of aspartate ligand, analogous to the active truncated insulin receptors cited above. It remains possible, however, that intracellular aspartate plays a stimulatory role in the initial activa-
tion and autophosphorylation of AIR. Indeed, we have occasionally observed 2-fold aspartate stimulation of AIR autophosphorylation
in membrane preparations (not shown). However, these observations have not been reproducible so further evaluation of reaction conditions will be necessary to verify such an effect.

Acknowledgments—We thank C. Trueblood for valuable advice with immuno-fluorescence and L. England for essential assistance with phos-
photyrosine Western blotting.

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FIG. 5. DNA synthesis in CA3 and parental cells measured by [3H]thymidine incorporation into serum-starved and -stimulated cells. Serum-starved cells were stimulated for 18 h with 10% calf serum (+); unstimulated cells received no addition (−). Determinations of incorporated tritium were from triplicate wells and had standard deviations below 10%.

Evidently the foreign extracellular sequences present in AIR and the absence of α subunit glycosylation sites do not prevent the expression of a kinetically functional receptor but do affect its intracellular transport. These examples of endoplasmic reticulum retention are consistent with prevailing views of trafficking of receptors in general (22, 23) and the IR in particular (24). Insulin receptors lacking most of the α subunit sequences and that are therefore unable to bind insulin have been shown to be constitutively activated (25–27). These observations have been interpreted as evidence that the IR ligand binding domain is inhibitory; binding or deletion of this domain apparently relieves an inhibition of the cytoplasmic kinase via a conformational change. Additionally, such truncated insulin receptors were not transported to the plasma membrane but were retained in the endoplasmic reticulum when expressed in Chinese hamster ovary cells (24). Our studies add to the existing data on compatibility of foreign sequences because AIR contains a procaryotic four-helix bundle domain flanked by two procaryotic transmembrane domains. These N-terminal do-
tains a procaryotic four-helix bundle domain flanked by two