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Retinoic acid alters the intracellular trafficking of the mannose-6-phosphate/insulin-like growth factor II receptor and lysosomal enzymes

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ABSTRACT **Previously, we showed that retinoic acid (RA) binds to the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) with high affinity, suggesting that M6P/IGF2R may be a receptor for RA. Here, we show that RA, after 2–3 h of incubation with cultured neonatal-rat cardiac fibroblasts, dramatically alters the intracellular distribution of M6P/IGF2R as well as that of cathepsin B (a lysosomal protease bearing M6P). Immunofluorescence techniques indicate that this change in intracellular distribution is characterized by a shift of the proteins from the perinuclear area to cytoplasmic vesicles. The effect of RA was neither blocked by an RA nuclear receptor antagonist (AGN193109) nor mimicked by a selective RA nuclear-receptor agonist (TTNPB). Furthermore, the RA-induced translocation of cathepsin B was not observed in M6P/IGF2R-deficient P388D1 cells but occurred in stably transfected P388D1 cells expressing the receptor, suggesting that the effect of RA might be the result of direct interaction with M6P/IGF2R, rather than the result of binding to the nuclear receptors. These observations not only support the idea that M6P/IGF2R mediates an RA-response pathway but also indicate a role for RA in control of intracellular trafficking of lysosomal enzymes. Therefore, our observations may have important implications for the understanding of the diverse biological effects of retinoids.**

The mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) is a 300-kDa multifunctional transmembrane glycoprotein. Recently, this receptor has been shown to play a fundamental role in the control of cell growth in fetal development (1–3) and carcinogenesis (4–10).

M6P/IGF2R is primarily (90–95%) localized in the intracellular compartments, particularly in the trans-Golgi network (TGN) and endosomes, with 5–10% of the receptor present on the cell surface (11). The primary function of M6P/IGF2R is to sort and transport M6P-bearing glycoproteins from TGN to endosomes/lysosomes (11). Normally, the newly synthesized soluble lysosomal enzymes are posttranslationally modified to contain M6P residues on their N-linked oligosaccharides. The acquired M6P residues enable the enzymes to bind to M6P receptors in the TGN. The receptors and their ligands cluster into clathrin-coated transport vesicles and travel to an acidic prelysosomal compartment where the low pH causes dissociation of the receptor–ligand complex. The free M6P receptors can travel to the plasma membrane or back to the TGN to reinitiate another cycle of biosynthetic enzyme transport. In the absence of M6P receptors, most M6P-containing glycoproteins are secreted from the cell. Thus, M6P/IGF2R is important for the intracellular retention of lysosomal proteins and their targeting to lysosomes. In addition, M6P/IGF2R can bind extracellular ligands, mediate endocytosis of IGF2 (12), and participate in the activation of latent transforming growth factor β (13).

Interestingly, our recent work shows that M6P/IGF2R also directly binds retinoic acid (RA) with high affinity (14). The binding site for RA is distinct from those for M6P and IGF2 on the receptor. A positive cooperativity exists between M6P and RA binding to M6P/IGF2R, as evidenced by the fact that binding of one compound (RA or M6P) enhances binding of the other (14). Furthermore, RA could increase the M6P-inhibitable endocytosis of β-glucuronidase and induce a transient intracellular accumulation of acid phosphatase (a M6P-containing lysosomal enzyme) in cultured cardiac myocytes (14). These findings led us to speculate that RA, on binding to M6P/IGF2R, acts as a functional regulator of the receptor to facilitate binding and trafficking of lysosomal enzymes. However, direct evidence of an effect of RA on intracellular translocation of both M6P/IGF2R and lysosomal enzymes is needed to support this idea. Here, by using immunofluorescence techniques, we show that RA is indeed capable of altering the intracellular distribution of both M6P/IGF2R and lysosomal enzymes.

MATERIALS AND METHODS

Materials. RA was obtained from Sigma. The synthetic retinoids used (AGN193109 and TTNPB) were produced by Allergan (Irvine, CA). Stably transfected P388D1 cells reexpressing M6P/IGF2R were kindly provided by William S. Sly (St. Louis University, St. Louis). The rabbit polyclonal antibody against the rat M6P/IGF2R was a generous gift from R. G. MacDonald (University of Nebraska, Omaha). The rabbit polyclonal anti-rat cathepsin B antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch.

Cell Culture. Neonatal-rat cardiac fibroblasts were prepared from 1-day-old rats by using the Neonatal Cardiomyocyte Isolation System (Worthington) and grown on glass coverslips in F-10 nutrient mixture with 10% horse serum and 5% fetal bovine serum (FBS). Cells were used in experiments after 3–5 days of culture. Mouse macrophage P388D1 cells were cultured in minimum essential medium-α supplemented with 10% FBS.

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Abbreviations: IGF2, insulin-like growth factor II; IGF2R, IGF2 receptor; M6P, mannose-6-phosphate; RA, retinoic acid; RAR, retinoic acid nuclear receptor; TGN, trans-Golgi network.

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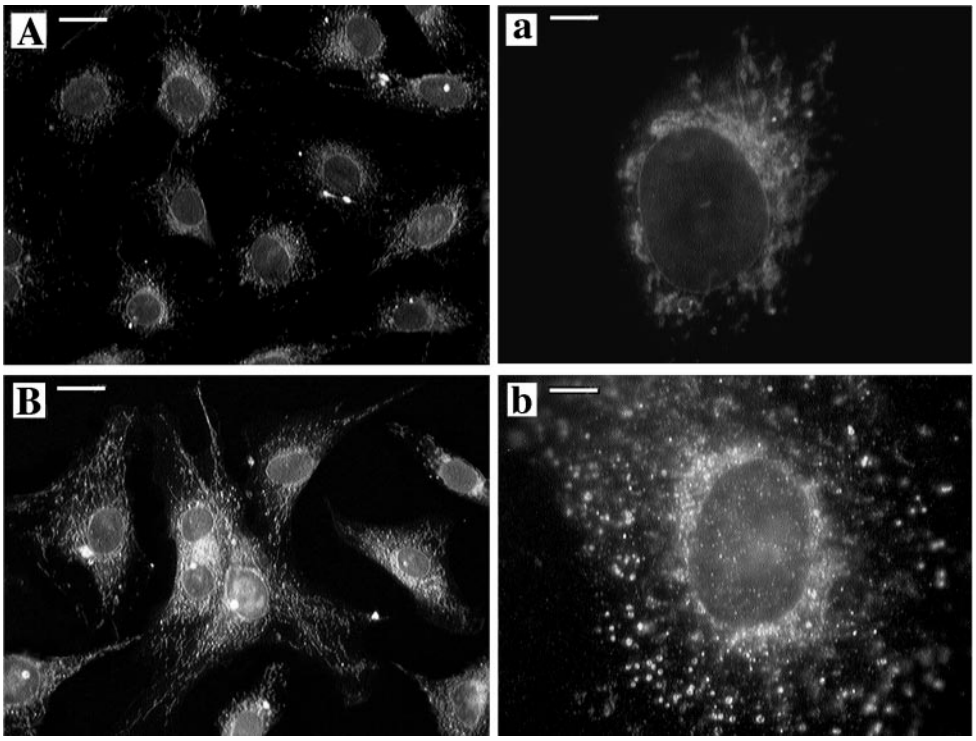

FIG. 1. Effect of RA on the distribution of M6P/IGF2R in neonatal-rat fibroblasts. (*A* and *a*) Intracellular localization of M6P/IGF2R in nontreated fibroblasts was detected by immunofluorescence with anti-M6P/IGF2R antibodies. The receptor was concentrated primarily in the perinuclear structure (probably the TGN area). (*B* and *b*) Distribution of M6P/IGF2R in RA-treated cells. After treatment with RA (1 μM for 3 h), the receptor translocated from the perinuclear area to cytoplasmic vesicles (possibly the endosomes and lysosomes). (Bars for *A* and *B* = 20 μm; bars for *a* and *b* = 4 μm.)

Immunofluorescence. Cells grown on glass coverslips for 3 days were treated with RA (0.25–1 μM) and/or synthetic retinoids for 2–3 h. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min. The cells were washed in PBS,

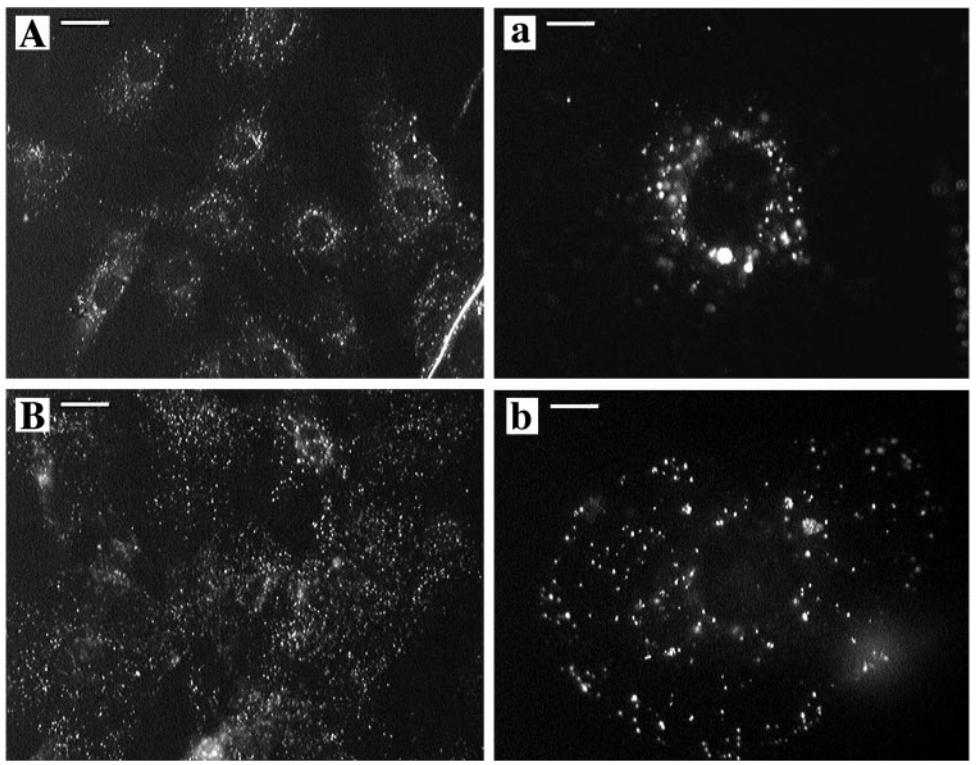

FIG. 2. Effect of RA on the distribution of cathepsin B in neonatal-rat fibroblasts. (*A* and *a*) Intracellular localization of cathepsin B in nontreated fibroblasts was detected by immunofluorescence with anti-cathepsin B antibodies. The enzyme was found mainly in the perinuclear area. (*B* and *b*) Distribution of cathepsin B in RA-treated cells. After treatment with RA (1 μM for 3 h), the enzyme translocated from the perinuclear area to the cytoplasmic vesicles. (Bars for *A* and *B* = 20 μm; bars for *a* and *b* = 4 μm.)

made permeable with 0.1% Triton X-100 in PBS for 5 min, and then incubated with either polyclonal anti-rat M6P/IGF2R antibody or anti-rat cathepsin B antibody (diluted 1:200) at room temperature for 1 h. This incubation was followed by washing in PBS and incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 h at room temperature. After washing in PBS, the cells were mounted on glass slides in an antifade mounting medium and photographed with a Nikon photomicroscope.

RESULTS AND DISCUSSION

To determine whether RA has an effect on intracellular trafficking of M6P/IGF2R, the distribution of the receptor in neonatal-rat fibroblasts with or without RA treatment was visualized by immunofluorescence. The primary antibody used to target the receptor was a polyclonal anti-rat M6P/IGF2R (full-length) rabbit IgG with high specificity (ref. 14; IgG from an unimmunized rabbit was used as a negative control). As indicated by fluorescence (Fig. 1 *A* and *a*), normally M6P/IGF2R in the fibroblasts is concentrated primarily in the perinuclear structure (probably the TGN). When cells were treated with 0.25–1 μM RA for 2–3 h, the distribution of the receptor in the cells exhibited a dramatic change, characterized by a movement of the receptors from the perinuclear area to the cytoplasmic vesicles (probably the endosomes and lysosomes) throughout the entire cytoplasm of the cell (Fig. 1 *B* and *b*), indicating a potentiating effect of RA on the trafficking of M6P/IGF2R from the TGN to the endosomes/lysosomes.

Because biosynthetic transport of lysosomal enzymes from the TGN to endosomes/lysosomes is mediated mainly by M6P/IGF2R (15), enhanced trafficking of the receptor should lead to increased translocation of the M6P-containing lysosomal enzymes from the TGN to lysosomes. To test this hypothesis, we examined the effect of RA on the intracellular distribution of cathepsin B, a lysosomal protease known to be transported in an M6P-dependent manner in fibroblasts (16). As expected, treating the cells with RA resulted in a remarkable change in cathepsin B distribution (Fig. 2), similar to that of M6P/IGF2R (i.e., cathepsin B shifted from the perinuclear area to the cytoplasmic vesicles). Thus, we have been able to show that RA can induce intracellular translocation of both M6P/IGF2R and lysosomal enzymes.

Next, it was essential to determine whether the observed effect of RA is mediated by the nuclear RA receptors (RARs). Therefore, we tested whether the effect of RA on M6P/IGF2R and cathepsin B trafficking could be blocked by a potent RAR antagonist and whether the RA effect could be mimicked by a potent RAR agonist. TTNPB, a very potent RAR agonist (17–21), and AGN193109, a RAR antagonist able to block the action of RA (mediated by RARs) completely when used at a 10-fold molar excess (19–21), were selected for use in these experiments. As shown in Fig. 3, the effect of RA on the distribution of either M6P/IGF2R or cathepsin B was neither blocked by the RAR antagonist nor mimicked by the RAR agonist. These results suggest that the action of RA in the translocation of M6P/IGF2R and cathepsin B is independent of RARs.

To test further the idea that M6P/IGF2R acts as an RA receptor that mediates the effect of RA on lysosomal-enzyme trafficking, we examined the effect of RA on the distribution of cathepsin B in cells that either lack or overexpress M6P/IGF2R. Mouse macrophage P388D1 cells are the best characterized cells that lack M6P/IGF2R (22, 23). Transfer of the M6P/IGF2R gene into these cells has created a population of P388D1 cells that stably overexpress the receptor (23). The responses of these two different populations of P388D1 cells (with and without M6P/IGF2R) to RA-induced cathepsin B translocation are shown in Fig. 4. No significant effect of RA was found in the M6P/IGF2R-deficient cells, whereas a re-

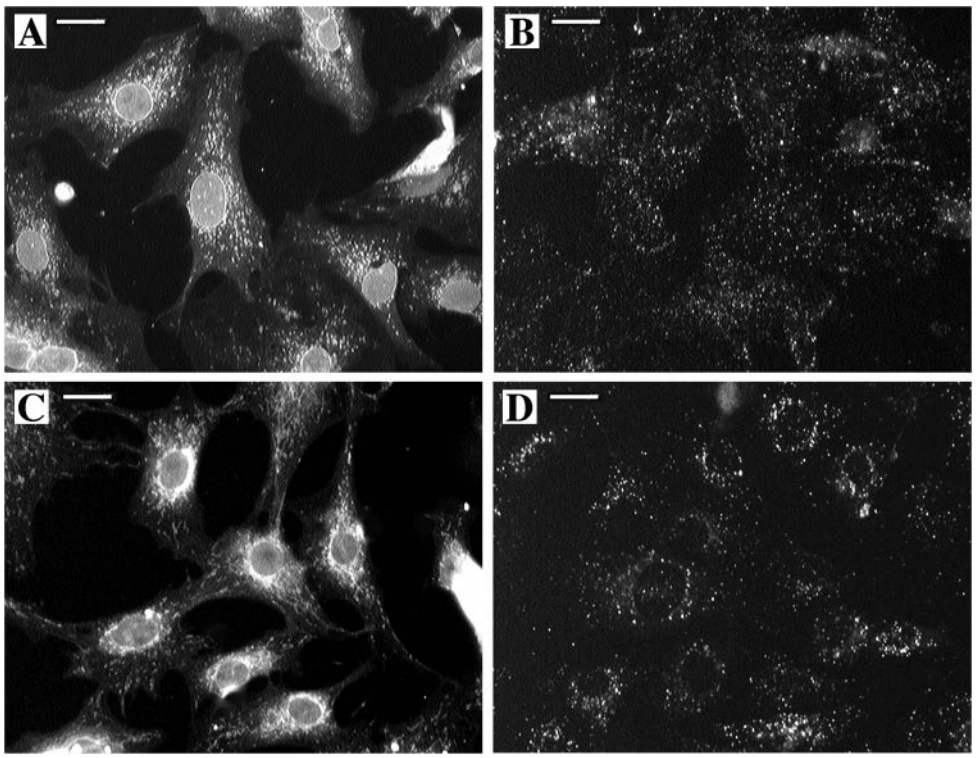

FIG. 3. Effects of RAR agonist and RAR antagonist on RA-induced translocation of M6P/IGF2R and cathepsin B. Cultured neonatal-rat cardiac fibroblasts were treated either with 0.25 μM RA and 3 μM AGN193109 (RAR antagonist; *A* and *B*) or with 1 μM NNTPB alone (RAR agonist; *C* and *D*) for 3 h. Distribution of M6P/IGF2R (*A* and *C*) and cathepsin B (*B* and *D*) was detected by immunofluorescence with anti-M6P/IGF2R antibodies and anti-cathepsin B antibodies, respectively. The RAR antagonist failed to block the effect of RA, and the RAR agonist did not mimic the effect of RA shown in Figs. 1 and 2. (Bars = 20 μm.)

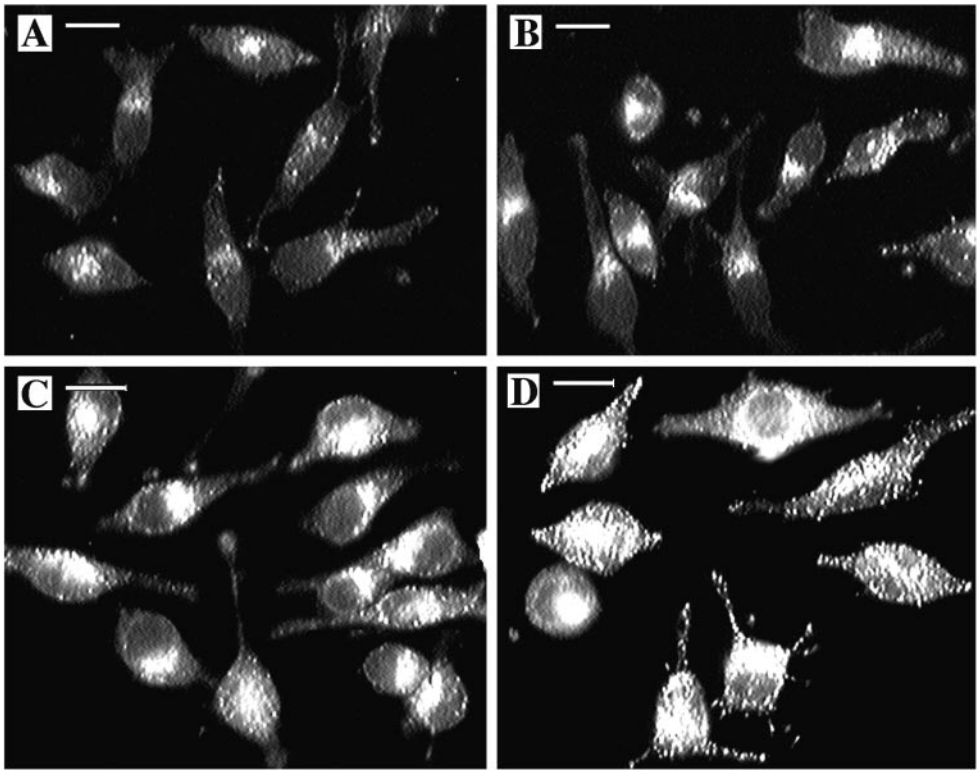

FIG. 4. Effect of M6P/IGF2R on RA-induced translocation of cathepsin B in P388D1 cell lines. The M6P/IGF2R-deficient P388D1 cells (*A* and *B*) or stably transfected P388D1 cells (reexpressing M6P/IGF2R; *C* and *D*) were treated either with (*B* and *D*) or without (as a control; *A* and *C*) 1 μM RA for 3 h. Intracellular distribution of cathepsin B was detected by immunofluorescence. RA-induced translocation of cathepsin B from the perinuclear area to the entire cytoplasm occurred in the transfected cells but not in the receptor-deficient cells. (Bars = 20 μm.)

markable cathepsin B translocation, similar to that observed in the fibroblasts, was found in the stably transfected P388D1 cells overexpressing M6P/IGF2R. These results, therefore, suggest that M6P/IGF2R plays an important role in mediating the effects of RA.

The results presented here indicate that RA can alter intracellular distribution of M6P/IGF2R, leading to enhanced trafficking of newly synthesized lysosomal enzymes from the TGN to the endosomes/lysosomes. The RA-induced redistribution of M6P/IGF2R and the lysosomal enzymes seems to be independent of the action of RARs. Previously we observed that RA binds to M6P/IGF2R with high affinity and can induce a 2- to 3-fold increase in the binding and endocytosis of exogenous lysosome enzymes as well as an increase in the cellular activity (sorting) of endogenous lysosomal enzymes (14). These observations, together with the findings reported here, strongly support a direct biochemical interaction between RA and M6P/IGF2R that is functionally important in the regulation of lysosomal enzyme trafficking. However, the mechanism by which the binding of RA to M6P/IGF2R increases the trafficking of the receptor remains to be investigated.

In mammalian cells, there are numerous lysosomal enzymes with various functions. In addition to their lysosomal role, these enzymes may be involved in a variety of physiological and pathological processes. In most instances, lysosomal enzymes are synthesized as proenzyme forms of higher molecular size and are targeted by the M6P receptors to endosomes/lysosomes where they become activated. Some of the activated enzymes, in turn, can modify (activate) other proenzymes or prohormones (e.g., proinsulin, proalbumin, and proapolipoproteins) and degrade endocytosed macromolecules or cellular components (e.g., IGF2). Obviously, the biosynthetic transport (trafficking of newly synthesized enzymes from the TGN to the endosomes/lysosomes) is a critical process that determines the bioavailability and activity of many cellular enzymes, including such proteases as cathepsin B and D, known to be involved in the induction of apoptosis (24–26). It is conceivable that any agent capable of altering this process (i.e., the trafficking function of the M6P receptors) could have a profound impact on cellular functions, including growth and death. Therefore, our findings (that RA binds to M6P/IGF2R and alters its trafficking function) make available retinoic actions that may be important in cell-function regulation and may lead to a better understanding of how retinoids are able to elicit an enormous diversity of biological responses.

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