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THE G_{M2} ACTIVATOR PROTEIN DOES NOT PLAY A CRITICAL ROLE IN ENDOSOME AND LYSOSOME MEMBRANE FUSION

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A recent report (Kuwana, T., Mullock, B.M., and Luzio, J.P. (1993) Biochem Soc Trans 21, 299-300) presented data suggesting that the rat homologue of the human G_{M2} activator protein may stimulate the fusion of late endosomes with lysosomes. To evaluate this possibility we monitored the bulk endocytosis and lysosomal incorporation of horseradish peroxidase by normal human fibroblasts and by mutant fibroblasts which lack functional lysosomal G_{M2} activator protein. A comparison of the peroxidase as well as the endogenous hexosaminidase distributions between endosomal and lysosomal fractions from these two cell lines failed to reveal any quantitative differences. We conclude that the G_{M2} activator protein is not needed for efficient fusion of lysosomal and endosomal membranes *in vivo*.

G_{M2} ganglioside (GalNAcβ(1-4)-{NANAα(2-3)-}-Galβ(1-4)-Glc-ceramide), is degraded by the lysosomal hydrolase β-hexosaminidase A (Hex A). The *in vivo* hydrolysis requires the participation of the G_{M2} activator protein (activator). Biosynthetic data indicate a the activator is synthesized in the rough endoplasmic reticulum as a 24 kDa precursor (the pro-form) which is processed to its 22 kDa mature form in the lysosome (1). The only confirmed *in vivo* function of the activator is to transport G_{M2} ganglioside from the lysosomal membrane to Hex A for hydrolysis (rather than to activate Hex A). It thus acts as a substrate-specific cofactor for Hex A (2). A deficiency of the activator protein, or the α or the β subunits of Hex A can cause neuronal storage of the ganglioside and the three autosomal recessive inherited diseases collectively known as the G_{M2} gangliosidoses. The three forms of this disease are Tay-Sachs disease (α defects), Sandhoff disease (β defects), and the rare AB variant form of G_{M2} gangliosidosis (activator defects) (reviewed in (3)).

We (4) and others (5) have identified a mutation in the gene encoding the activator protein of an AB-variant patient. A $T^{412}C$ transition was found in the homozygous form in

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cDNA and genomic DNA from the patient. This nucleotide change results in the substitution of Cys^{138} with an Arg residue in the activator protein. Whereas the patient's fibroblasts produce apparently normal levels of activator mRNA, they lack functional lysosomal activator protein. Lysates from COS-1 cells transfected with a mammalian expression vector containing the wild type activator cDNA produced an elevated level of both pro- and mature forms of the activator protein, with an accompanying eleven-fold enhancement in the ability of purified Hex A to hydrolyze G_{M2} ganglioside. However, lysates from cells transfected with the vector containing the mutant ($Cys^{138}Arg$) cDNA construct contained only low levels of the pro-activator protein which failed to enhance Hex A activity significantly above the endogenous level of mock transfected COS cells (4).

The pathological descriptions of the rare AB-variant form are limited to two autopsy cases. Although there were numerous parallels with Tay-Sachs and Sandhoff disease, the observation of "prominent inclusions in glial cells, including large conglomerates of lipid inclusions", were significantly different (3). This may suggest some other function(s), as yet uncharacterized for the activator. One such candidate function was recently proposed by Kuwana *et al.* (6). They used a fluorescence-dequenching assay to monitor endosomal-lysosomal fusion events *in vitro*. They identified a 22kDa protein whose purification from a low-salt extract of rat-lysosomes and final elution profile from a HPLC gel filtration column, coincided with the originally observed fluorescence-dequenching activity of the extract. The N-terminal sequence (20 residues) of this protein was determined and found to be homologous to the N-terminus of the human activator protein. This sequence data plus the molecular weight and lysosomal localization data strongly suggest that the protein they isolated is the rat homologue of the human activator protein. Since the human activator has been shown to function as a ganglioside-transfer protein *in vitro* (7), the authors were unable to determine if the protein was functioning in their fluorescence-dequenching assay as a true fusogen or simply as a more general lipid-transfer protein (6). In the present report we utilize our well characterized AB-variant fibroblast cell line (above) to determine if the lack of functional lysosomal activator results in an abnormal distribution, between the late endosomal and dense lysosomal compartments, of the endogenous lysosomal hexosaminidase or newly endocytosed horseradish peroxidase.

MATERIALS AND METHODS

Experiments were performed according to Wilson et al.(8). Normal and AB variant (4) fibroblasts were grown in α-MEM with 10% fetal calf serum in 200 mm dishes until they reached confluence. The cells were split 2:1 and the next day incubated for 1.5 h in serum-free α-MEM containing 2.5mg/ml horseradish peroxidase (Sigma, Type II). The cells were washed 5 times with ice-cold α-MEM and once with HB (250mM sucrose, 2mM EDTA, 10mM Hepes, pH7.4), and scraped with a rubber policeman into HB. Scraped cells were centrifuged for 10 min. at 800g, resuspended in 4mL HB, and homogenized with 10 strokes of a glass Dounce homogenizer. The homogenates were centrifuged for 10 min. at 800g and the supernatants were mixed with HB and Percoll (Pharmacia) to produce 11mL of a solution containing 27% Percoll. The mixtures were transferred to Beckman centrifuge tubes which were then completely filled by the addition of 60% sucrose (~1.5mL). The tubes were sealed and centrifuged at 20,000 rpm for 1 h in the Beckman VTi 65.1 rotor. Approximately 0.5mL fractions were collected (the exact

"volumes" were determined by the difference in weight of each tube before and after filling) from a needle hole at the bottom of each tube with the flow controlled by air-pressure introduced by a needle connected with a peristaltic pump inserted at the top of the tube. From each 500μL fraction, 20μL was taken to test the hexosaminidase activity using the 4-methylumbelliferyl substrate (9), and 200μL was taken to assay for horseradish peroxidase activities using the BioRad "Peroxidase Substrate Kit" (catalog # 172-1064). In the former case activity was recorded as relative fluorescence units and in the latter case as relative reaction rates ($1000X\Delta OD_{415}$/sec).

RESULTS AND DISCUSSION

We tested the AB-variant cell line's ability to internalize and accumulate in dense lysosomal compartments the fluid-phase marker horseradish peroxidase (exogenously added to theculture medium) (10). The postnuclear supernatants of normal and mutant fibroblast homogenates were separated on a 27% Percoll gradient. This gradient has been shown to separate endosomes (both early and late) from dense lysosomes (8, 11). Since the biosynthetic pathway for most lysosomal enzymes involves passage from the Golgi to the late endosome and finally the lysosome (reviewed in (12)), and there is evidence that lysosomal enzymes may normally be recycled back to endosomes (10), each 0.5mL fraction (taken from the bottom of the

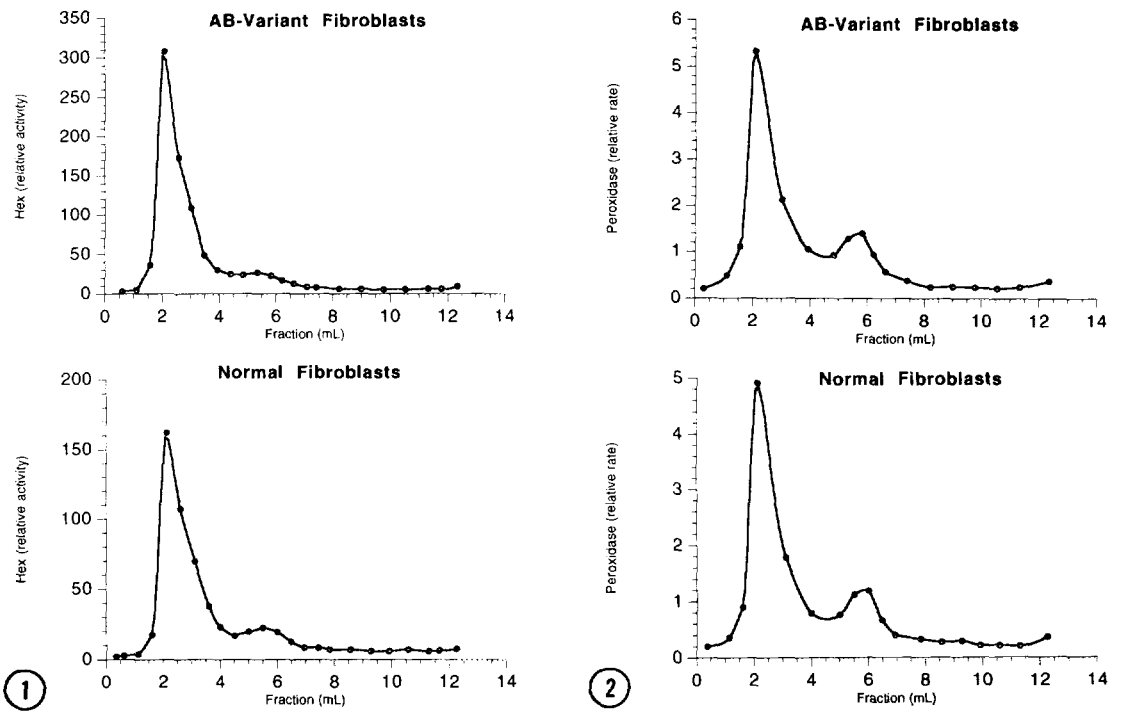

Figure 1. The separation on a 27% Percoll gradient into lysosomes (large peak, near the bottom of the tube) and endosomes (both early and late, smaller peak) of the postnuclear supernatants from normal and AB-variant fibroblast homogenates. The elution volumes were plotted against total hexosaminidase (endogenously synthesized) activity expressed as relative fluorescence units.

Figure 2. The same two sets of fractions described in the legend to Fig. 1 were assayed for the presence of the fluid-phase marker horseradish peroxidase (internalized from the medium and accumulated in lysosomal and endosomal compartments). Fraction volumes were plotted against the reaction rate, $1000X\Delta OD_{415}$/sec (relative rate).

tube) was assayed for total endogenous hexosaminidase activity (Fig. 1), as well as for internalized horseradish peroxidase activity (Fig. 2). In both cases the activities contained in the homogenates of each cell line were separated by the gradient into one large peak, corresponding to the dense lysosomes (bottom of the tube), and one smaller peak, corresponding to the endosomes. The distribution of the two activities between the endosomal and lysosomal fractions of AB-variant and normal fibroblasts were nearly identical (Fig. 1 and 2). Thus, neither the intracellular transport of newly synthesized hexosaminidase nor fluid-phase endocytosis and lysosomal incorporation of horseradish peroxidase are inhibited in the AB-variant cells. Thus, these data suggest that there is no inhibition of endosomal/lysosomal fusion in the AB-variant cells.

The above data indicate that the activator protein does not play a role *in vivo* in endosomal and lysosomal membrane fusion. They are consistent with the activator causing the fluorescence-dequenching activity observed by Kuwana *et al.* (6) through its ability to transfer lipids. However, these data do suggest that the observed *in vitro* transfer of glycolipids between liposomes by the activator (7) may reflect an uncharacterized *in vivo* activator-function involving lipid transfer between endosomal and lysosomal membranes.

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