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# INHIBITORS OF GLYCOPROTEIN PROCESSING ACT AT AN EARLY STAGE OF MYOGENESIS

Geralyn C. Trudel and Paul C. Holland[1]

Muscle Biochemistry Laboratories, Montréal Neurological Institute
McGill University, Montréal, Que., Canada H3A 2B4

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SUMMARY: The glycoprotein processing inhibitors bromoconduritol and N-methyl-1-deoxynojirimycin inhibit myoblast fusion and differentiation, suggesting the critical involvement of one or more glycoproteins in the control of skeletal myogenesis. In the present study we have examined the effect of inhibitors of glycoprotein processing on the expression of the muscle-specific regulatory factor myogenin. Glucosidase inhibitors, but not the mannosidase inhibitor 1-deoxymannojirimycin, inhibited the accumulation of myogenin mRNA in myoblasts, and immunoblotting confirmed that this was reflected in reduced accumulation of myogenin protein. The results indicate that the glycoprotein(s) critically involved in the control of myoblast differentiation act at an early stage in this process by modulating expression of the myogenic regulatory factor myogenin. 

Several studies indicate that one or more N-linked glycoproteins are critically involved in the control of skeletal muscle differentiation. Thus, concanavalin A-resistant skeletal myoblast cell lines are unable to fuse or biochemically differentiate (1,2) and tunicamycin and inhibitors of the glucosidases involved in glycoprotein processing also inhibit myoblast fusion and differentiation (3-7).

The glycoprotein, or glycoproteins, involved in the control of myogenesis have not yet been unequivocally identified. Evidence has been presented for a critical role of an integrin in avian myoblast differentiation (8) in support of which we have shown that inhibitors of processing glucosidases, which affect myogenesis, also inhibit the translocation of integrin subunits to the myoblast cell surface (7,9).

[1] Author to whom reprint requests should be addressed.

ABBREVIATIONS

BCD; 6-bromo-3,4,5, trihydroxy-cyclohex-1-ene, HS; horse serum, MDJN; N-methyl-1-deoxynojirimycin, ManDJN; 1-deoxymannojirimycin.

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The precise stage of terminal differentiation at which the glycoprotein(s) affected by glucosidase inhibitors is involved is also unknown. A family of nuclear proteins has recently been identified, members of which can activate both the determination of cells into the myogenic lineage, and the terminal differentiation of cells already committed to the myogenic lineage (10-14). The most fully studied of these factors are $MyoD_1$, (10), and myogenin, (11).

Cells of the rat $L_6$ myoblast cell line express myogenin but not $MyoD_1$. Recent studies in this cell line, using myogenin antisense oligonucleotides to block the accumulation of myogenin mRNA (15) have demonstrated that myogenin expression controls $L_6$ myoblast terminal differentiation. To gain further insight into the precise stage at which glycoproteins are critically involved in the control of myogenesis we have used the $L_6$ myogenic system to determine the effect of inhibitors of glycoprotein processing on myogenin expression.

The results demonstrate that inhibitors of glycoprotein processing which inhibit myoblast differentiation markedly reduce the accumulation of myogenin protein and mRNA, and suggest that cell-surface glycoproteins are critically required at an early stage of myogenesis to transduce a signal leading to increased myogenin gene expression.

MATERIALS AND METHODS: Rat $L_6$ myoblasts were a sub-clone of the line originally isolated by Yaffe (16) and were cultured as previously described (7). Mouse C2 myoblasts (17) were obtained from Dr. Bruce Paterson, National Cancer Institute, Bethesda, MD, USA. DMEM was obtained from Gibco, Grand Island, N.Y., U.S.A; HS was obtained from Flow Labs, McLean, Virginia. BCD, ManDJN, MDJN, agarose, restriction enzymes, random prime labelling kit, phenol, acrylamide, SDS, TEMED and Tris were all obtained from Boehringer Mannheim Canada, Laval, Que., Canada. [$\alpha$-$^{32}$P]-dCTP and rainbow molecular weight markers were from Amersham Corp., Oakville Ont., Canada; Agarose was from BRL, Gaithersburg, MD, USA; ethidium bromide was from Sigma, St.Louis, MO, USA; Nytran, charge modified membrane was from Schleicher and Schuell, Keene, NH, USA. BCA protein assay kit was from Pierce; ABC-AP kit was from Dimension Labs., Mississauga, Ont., Canada. All other chemicals were obtained from Fisher Canada, Montreal, Que., Canada.

cDNA Probes: Plasmid pUC65-2 containing the complete coding sequence for myogenin (11) and anti-myogenin monoclonal antibody (18) were provided courtesy of Dr. W. Wright, Department of Cell Biology, University of Texas, Southwestern Medical School, Dallas, TX., USA. Plasmid pVZC11$\alpha$ containing the full-length $MyoD_1$ coding sequence was provided courtesy of Drs. Lassar and Weintraub, Hutchinson Cancer Research Center, Seattle, Washington, USA. Plasmid pHM$\alpha$A-1 containing the probe LK248 (for actin) was provided courtesy of Dr. L. Kedes, Veterans Administration, Palo Alto, CA, USA.

Isolation of RNA: Cell monolayers were extensively washed in PBS, and solubilized in 0.5% SDS/ 50mM Tris-HCl (7.5 pH)/1mM EDTA. RNA was isolated from cultured myoblasts by two phenol extractions followed by a chloroform extraction, and ethanol precipitation overnight, repeated twice. This was followed by LiCl precipitation by the method of Sambrook, Fritsch and Maniatis, (19).

Northern Blotting: Total RNA samples were separated by gel electrophoresis in 1.5% agarose and 7% formaldehyde, then transferred by capillary action to Nytran (charge modified membrane)

over an 18 h period. RNA blots were UV cross-linked for a period of 1.5 min. Blots were incubated for a minimum of 6 h in a sealed plastic bag (air removed) in a pre-hybridization solution containing: 50% deionized formamide (v/v); 5x SET; 10x Denhardt's; salmon sperm DNA (final = [0.5 mg/ml]); 0.5% SDS (w/v), at 47°C, followed by probing with cDNAs as described below (19). Plasmids containing the myogenin and $MyoD_1$ inserts were cut with EcoR1 prior to random prime labelling with [$\alpha^{32}$P]-dCTP, and used immediately following labelling. Plasmid pHM$\alpha$A-1 (actin probe LK248) was cut with Xba 1 prior to labelling as indicated above. Pre-hybridization solution was removed from the plastic bag and the [$\alpha^{32}$P]-labelled cDNA probe (100 ng) was added in a hybridization solution containing: 50% deionized formamide (v/v); 5x SET; 1x Denhardt's; salmon sperm DNA (final= [0.1 mg/ml]); 0.5% SDS (w/v), 17% dd $H_2O$, at 47°C. Following labelling blots were washed with either high stringency buffer, (0.1% SSC, 0.1% SDS, 65°C) or moderate stringency buffer, (1.0% SSC, 0.1% SDS, 47°C) as indicated in the results and were exposed to film.

Western Blotting: $L_6$ myoblasts were grown for 8 days with or without the glucosidase inhibitor BCD added to a final concentration of 10µg/ml each day on days 1 through 5. Cells were harvested at the time points indicated in the results. Samples were run on a 10% SDS-PAGE gel and electrophoretically transferred to nitrocellulose (20). Nitrocellulose blots were incubated in BLOTTO (21) and overlaid with anti-myogenin monoclonal antibody followed by alkaline phosphatase-conjugated secondary antibody (as per manufacturers instructions).

## RESULTS

As reported by others (11,22) northern blotting of total RNA from rat $L_6$ myoblasts indicated these cells express myogenin but not $MyoD_1$ mRNA. The $MyoD_1$ probe did however react with RNA isolated from C2 myoblasts probed at the same time and run on the same gel (Fig. 1). $L_6$ myoblasts therefore represent a system in which terminal differentiation may be predominantly controlled by the regulatory factor myogenin.

We measured myogenin mRNA accumulation in $L_6$ myoblasts grown in the presence of inhibitors of glycoprotein processing. The levels of myogenin mRNA in $L_6$ myoblasts treated with the glucosidase inhibitors bromoconduritol (BCD), or N-methyl-1-deoxynojirimycin (MDJN), were markedly decreased (Fig. 2, lanes 2 and 4 respectively) compared with control, untreated, myoblasts (Fig. 2, lane 3). In contrast myogenin mRNA accumulation was essentially unaffected by growth in the mannosidase inhibitor, 1-deoxymannojirimycin (ManDJN; Fig. 2 lane 1), which has no significant effect on myoblast fusion and differentiation (3,4,6). The blot was stripped and reprobed with LK248 probe directed against actin (Fig. 2). LK248 probe reacts with mRNA coding for both muscle ($\alpha$) and non-muscle ($\beta/\gamma$) actin (23). Probing with this marker served a dual purpose in that; (i) the signal for non-muscle ($\beta/\gamma$)) actin indicates that equal amounts of RNA were loaded in each lane of Fig. 2; (ii) the signal for the muscle-specific form of actin ($\alpha$-actin), which is a marker of myoblast differentiation (24), indicates that both MDJN-treated and BCD-treated myoblasts show impaired differentiation compared to control or ManDJN-treated myoblasts.

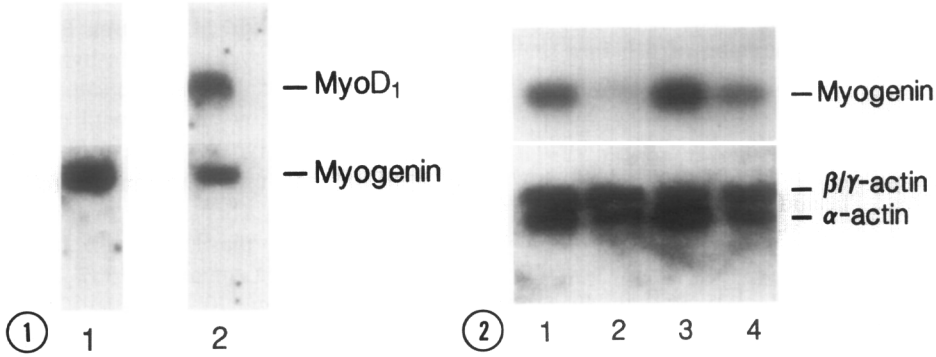


**Figure 1. Northern Blot of $L_6$ and C2 RNA probed with Myogenin and $MyoD_1$:** Total cellular RNA was isolated from $L_6$ (lane 1) and C2 cells (lane 2), just prior to fusion and separated on gels as described in materials and methods and probed with [$\alpha$-$^{32}$P]-dCTP labelled myogenin-cDNA. The blot was then stripped and reprobed with [$\alpha$-$^{32}$P]-dCTP labelled $MyoD_1$-cDNA. Note that C2 and $L_6$ both express myogenin mRNA (1.8 kb), while only C2 express $MyoD_1$ mRNA (1.8 kb). The size of myogenin and $MyoD_1$ mRNA was determined by migration of 28S and 18S rRNA.

**Figure 2. Northern Blot of $L_6$ RNA Probed with Myogenin or Actin cDNA:** $L_6$ total cellular RNA was isolated from untreated cells and cells treated with ManDJN (1mM), MDJN (1mM), or BCD (0.25mM) for 3 days. Samples were separated on gels as described in materials and methods, followed by northern blotting. Probing with [$\alpha$-$^{32}$P]-dCTP labelled myogenin-cDNA indicates reduced levels of myogenin mRNA (1.8 kb) in either BCD-treated (lane 2) or MDJN-treated (lane 4) cells compared to control (lane 3) or ManDJN-treated cells (lane 1). The blot was stripped and reprobed with [$\alpha$-$^{32}$P]-dCTP labelled actin cDNA (LK248). Note the equal amounts of $\beta/\gamma$-actin (2.5 kb) in lanes 1-4 and reduced amounts of muscle specific $\alpha$-actin (1.9 kb) in both BCD-treated (lane 2) or MDJN treated (lane 4) $L_6$ compared to untreated cells (lane 3) or ManDJN (lane 1).

To verify that the effect of glucosidase inhibitor on myogenin mRNA accumulation was reflected in reduced accumulation of the corresponding protein, we analyzed myogenin protein accumulation in control and BCD-treated cultures. This was done at several different stages of growth to establish that the inhibitor was not simply delaying the onset of myogenin induction but was effectively suppressing myogenin production throughout the course of incubation over an 8 day period.

Myogenin was detected on Western blots as a doublet with the major immunoreactive band at an apparent molecular weight of ~40 kDa and a minor band of ~42 kDa (Fig. 3). A similar myogenin doublet has been detected in mouse C2 myoblasts by Brennan and Olson (25), who suggested that the doublet represents differently phosphorylated forms of the protein. The lower apparent molecular weight of mouse myogenin (32 and 34 kDa) observed by Brennan and Olson (25) is consistent with the fact that the mouse sequence is 41 amino acid residues shorter than the rat myogenin sequence (26). In untreated myoblasts myogenin was barely detectable at 2 days after plating but its cellular concentration increased markedly by 3 days and continued increasing

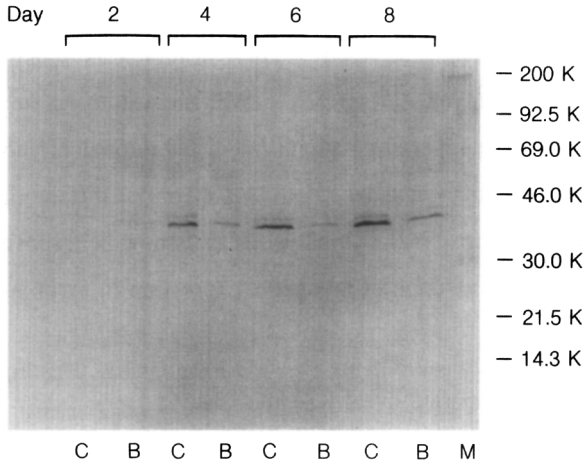


**Figure 3. Effect of the Glucosidase Inhibitor BCD on Myogenin Protein Expression in $L_6$ Cells:** The levels of myogenin (~40kD) were measured at days 2,4,6, and 8 in untreated (C) or BCD-treated (B) $L_6$ cells as described in methods, by immunoblotting with anti-myogenin monoclonal antibody. Myogenin expression is significantly reduced in the presence of the glucosidase processing inhibitor BCD. Molecular weight markers (lane M) are shown to the right of the figure.

through to the last time point measured at 8 days after plating. In cells treated with BCD only a very modest increase in myogenin accumulation was detectable even at day 8. Correspondingly, by 8 days after plating, control untreated cultures were over 80% fused whereas the BCD-treated cultures were under 20% fused (not shown).

## DISCUSSION

In rat $L_6$ myoblasts and BC3H-1 myoblasts, both of which express myogenin but not $MyoD_1$, it has been shown that inhibition of myogenin mRNA accumulation by the growth of cells in myogenin antisense oligonucleotides prevents myoblast terminal differentiation (15,27). This suggests that in such $MyoD_1$-deficient cells terminal differentiation is dependent upon myogenin expression. The process of terminal differentiation in $L_6$ myoblasts therefore requires two main events the first being expression of the regulatory factor myogenin and the second being downstream actions of myogenin on the activity of muscle-specific promotors. It has been known for some time that glycoproteins play a critical role in $L_6$ myoblast terminal differentiation (28) but the precise stage at which they are involved is unknown. In the present study we have found that inhibitors of glycoprotein processing affect the first phase of $L_6$ myoblast terminal differentiation defined above, namely, the accumulation of myogenin mRNA and protein.

Myogenin contains a single potential glycosylation site at residues 161-163 (11) but it is not known whether post-translational modification occurs at this site. If N-glycosylation does occur at this site the glucosidase inhibitors could modify myogenin function as a direct

consequence of inhibition of glycoprotein processing. Such a mechanism could not however explain the primary observation of the present study, that inhibitors of processing glucosidases inhibit the accumulation of myogenin mRNA. We suggest therefore that the action of glucosidase inhibitors on myogenin accumulation is mediated through effects of the inhibitors on cell-surface glycoproteins implicated in the control of myoblast differentiation through signal transduction, such as the integrins (7-9). This hypothesis is now being investigated by analysis of the role of integrins in the induction of myogenin expression in myoblasts.

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