p204 PROTEIN OVERCOMES THE INHIBITION OF THE DIFFERENTIATION OF P19 MURINE EMBRYONAL CARCINOMA CELLS TO BEATING CARDIAC MYOCYTES BY Id PROTEINS.

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SUMMARY
We reported in an accompanying article that (i) the p204 protein is required for the differentiation of murine P19 embryonal carcinoma stem cells to beating cardiac myocytes, and (ii) the expression of p204 in the differentiating P19 cells is synergistically transactivated by the cardiac transcription factors Gata4, Nkx2.5 and Tbx5. Here we report that endogenous or ectopic Id (Inhibitor of differentiation) proteins inhibited the differentiation of P19 cells to myocytes. This was in consequence of the binding of Id1, Id2 or Id3 protein to the Gata4 and Nkx2.5 proteins and the resulting inhibition (i) of the binding of these transcription factors to each other and to DNA, as well as (ii) of their synergistic transactivation of the expression of various genes including atrial natriuretic factor and Ifi204 (encoding p204). p204 overcame this inhibition by Id proteins in consequence of (i) binding and sequestering Id proteins, (ii) accelerating their ubiquitination and degradation by proteasomes, and (iii) decreasing the level of Id proteins in the nucleus by increasing their translocation from the nucleus to the cytoplasm. (ii) and (iii) depended on the presence of the Nuclear Export Signal in p204. In the course of the differentiation Gata4, Nkx2.5 and p204 were components of a positive feedback loop. This arose in consequence of it that p204 overcame the inhibition of the synergistic activity of Gata4 and Nkx2.5 by the Id proteins.

INTRODUCTION
p204 and p202a (which was designated earlier as p202) are the best characterized members of the interferon-inducible murine p200 family proteins (1-4). p202a modulates transcription, cell proliferation and apoptosis primarily by binding and modulating the activity of various sequence-specific transcription factors (5-9). p204, a sister protein of p202a, is also growth inhibitory (1,10,11). It can inhibit the transcription of ribosomal RNA by binding the ribosomal RNA-specific UBF transcription factor and inhibiting its binding to DNA (12). p204, similarly to p202a, contains the pRb, p107 and p130 protein binding motif LXCXE, and can bind these pocket proteins (12-14). p204 is involved in the differentiation of bone osteoblasts and skeletal muscle myoblasts (15-17).

During the differentiation of cultured C2C12 mouse skeletal muscle myoblasts to myotubes the p204 (and p202a) levels increased several fold in consequence of the transactivation of the Ifi204 gene by the muscle specific MyoD, myogenin and E12/E47 transcription factors (16).
Experiments, involving the use of 204 antisense (204AS) RNA for decreasing the level of endogenous p204, revealed that p204 is required for the differentiation of C2C12 myoblasts to myotubes (16). p204 enables the differentiation at least in part by overcoming the inhibition of the activities of MyoD, E12/E47 and other myogenic basic region-helix-loop-helix (bHLH) transcription factors by the Id (inhibitor of differentiation) (Footnote 1) proteins Id1, Id2 and Id3 (17). The Id proteins block skeletal muscle differentiation by binding the myogenic bHLH transcription factors, and inhibiting their binding to DNA (18-22). p204 overcomes this inhibition by binding and sequestering the Id proteins, and also by decreasing their levels (17).

We reported in an accompanying article that: (i) p204 is required for the differentiation of murine P19 embryonal carcinoma stem cells to beating cardiac type myocytes, and (ii) the expression of p204 during the differentiation is synergistically transactivated in the P19 cells by the cardiac Gata4, Nkx2.5 and Tbx5 transcription factors (23).

Whereas skeletal muscle differentiation involves the fusion of single myoblast cells to multinucleated myotubes, cardiac muscle consists of single myocytes (16,24). Furthermore, as noted earlier, the transcription factors involved in skeletal muscle differentiation differ from those functioning in cardiac muscle differentiation. In spite of these differences, however, the common involvement of p204 in skeletal muscle and heart muscle differentiation prompted us to explore whether the mechanisms of p204 actions are similar in the two processes.

As noted above, one of the functions of p204 in skeletal muscle differentiation is to overcome the inhibition of this process by the Id proteins. We tested whether the function of p204 in heart muscle differentiation can also be correlated with an involvement of Id proteins in the process.

Here we report that proliferating P19 cells contained Id1, Id2, and Id3. By binding to the cardiac Gata4 and Nkx2.5 transcription factors the Id proteins inhibited their synergistic activation of cardiac gene expression, and thereby blocked the differentiation of P19 cells to beating cardiac myocytes. The various mechanisms of inhibition by the Id proteins and the various mechanisms by which p204 overcomes these inhibitions are the topics of this study.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs.** To generate 204 expression plasmid constructs the full-length 204 cDNA was inserted into the EcoRI site of the pcDNA3 vector (Invitrogen), either in the proper orientation to obtain a p204 (sense) expression plasmid, (pCMV204), or in the opposite orientation to obtain a 204 antisense RNA expression plasmid (pCMV204AS) (1). The 204 cDNA segments inserted into both plasmids extended from nucleotide –127 to nucleotide +2226 (see Fig.2 in ref (1)). MBP-Gata4, various MBP-Gata4 segments, and MBP-Nkx2.5 were constructed in the pMAL-2 vector system (New England Biolabs). Gata4, Nkx2.5, Id1, Id2 and Id3 were each inserted into a pGBKKT7 yeast expression vector (Clontech) to generate pGBKKT7-Gata4 etc. Expression plasmids encoding Id3, GFP-Id3 and Flag-Id3 were constructed using the pcDNA3 vector, pEGFP-N1 vector, p3XFlag-CMV7.1 vector or p3XFlag-CMV14 vector (Sigma), respectively. p3XFlag-CMV7.1 linked the Flag moieties to the N terminus of Id3, p3XFlag-CMV14 to the C terminus. pcDNA-E1 was generated from the human E1 ubiquitin-activating enzyme expression plasmid pGEM-7ZF-E1 (25) (a gift from H. Zhang).

**Cell culture.** P19 cells were cultured and induced to differentiate as described (26). 10T1/2 cells, cloned murine embryonic fibroblasts (ATCC 226 CCL) (27), BLK cells (BLKSV HD. 2A. 5R. 1A. 3R. 1) mouse fibroblasts transformed with SV40 (ATCC TIB-88), and 293 cells human primary embryonal kidney cells transformed with adenovirus type 5 (ATCC CRL-1573) were cultured in DMEM supplemented with 10% FBS. Ind.p204 C2C12 cells (a line in which p204 expression can be induced by Muristerone (16)) were cultured in DMEM, 10% FBS. Human tsBN75 cells carrying a human thermosensitive E1 ubiquitin activating enzyme (a kind gift from C. Basilico) (28) were cultured in DMEM, 10% FCS at 33°C.
Coimmunoprecipitation. 293 cells cultured in 35mm dishes were cotransfected with pCGNGata4 (3\(\mu\)g) or pCGNNkx2.5 (3\(\mu\)g) as well as Flag-Id1 (3\(\mu\)g), Flag-Id2 (3\(\mu\)g) or Flag-Id3 (3\(\mu\)g) using the Lipofectamine 2000 reagent. Cells were harvested after incubation in DMEM, 10% FCS for 30 h and lysed in lysis buffer (29). 500 \(\mu\)g protein samples were immunoprecipitated by incubation with protein G beads, loaded with 10 \(\mu\)g of M2 monoclonal \(\alpha\)-Flag (Sigma), at 4°C overnight. The proteins retained on the loaded beads were washed and eluted following a published procedure (29). Western Blotting was conducted with \(\alpha\)-Gata4 or \(\alpha\)-Nkx2.5 as well as M2 monoclonal \(\alpha\)-Flag.

Binding of Gata4 to the Id proteins in vitro. Binding of purified MBP-Gata4 protein to purified GST-Id proteins in vitro. 500 \(\mu\)g of MBP-Gata4 or as control 500 \(\mu\)g of GST were incubated with 20 \(\mu\)l GST beads loaded with 500 \(\mu\)g of GST-Id1, GST-Id2, or GST-Id3, or as control GST in 300 \(\mu\)l AM-100 buffer (17) at 4°C, overnight. The beads were washed and eluted in SDS buffer by boiling. The MBP-Gata4 or MBP proteins retained on the loaded GST beads were assayed by Western Blotting using rabbit \(\alpha\)-MBP (NEB).

GST pulldown assay for Id2 domains and Gata4. 500\(\mu\)g of the indicated GST-Id2 segment or GST was loaded to 20 \(\mu\)l GST beads and was incubated with 10\(\mu\)l of \(^{35}\)S\]Gata4 (translated in vitro) in the same conditions as above. The retained \(^{35}\)S\]Gata4 was eluted and assayed by SDS-PAGE and autoradiography.

MBP pulldown assay for Gata4 segments and Id. Segments of Gata4 were fused to the C terminus of MBP. 500\(\mu\)g of each of the fusion proteins were bound to amylose beads (NEB) and were incubated with 10-15 \(\mu\)l of \(^{35}\)S\]labeled Id2 or Id3, (translated in vitro) in AM-100 buffer at 4°C overnight. Thereafter the bound protein complexes were eluted and assayed by SDS-PAGE and autoradiography.

Reporter gene assay. 10T1/2 cell cultures were grown and transfected in conditions described above. The cultures were cotransfected using Lipofectamine 2000 with 0.5\(\mu\)g reporter construct including the nucleotide –98 to +48 segment of the Ifi204 gene inserted into pGL3 vector (Promega) and 0.5\(\mu\)g pSVGal together with, if so indicated, 1\(\mu\)g pCGNGata4, 1\(\mu\)g pCGNNkx2.5, 2\(\mu\)g pcDNA3-Id1, 2\(\mu\)g pcDNA3-Id2, 2\(\mu\)g pcDNA3-Id3 and 3\(\mu\)g pCMV204. BLK cells were cotransfected using the calcium phosphate based mammalian transfection kit (Stratagene) with 1\(\mu\)g of a reporter construct in which the ANF gene segment (nucleotide –135 to +1) was inserted into the pGL3 vector, 1 \(\mu\)g pSVGal, and if so indicated 2\(\mu\)g pCGNGata4 and/or 2\(\mu\)g pCGNNkx2.5, 4 \(\mu\)g pCMV204 and 2, 4 or 6 \(\mu\)g of pCMV-Id3.

Biotinylated oligodeoxynucleotide precipitation assay. The same procedure for transfection as described above was used in 10T1/2 cells in 6 well plates. If so indicated 2 \(\mu\)g per well of pCMV-FlagId1, pCMV-FlagId2 or pCMV-FlagId3 without or with 0.5 \(\mu\)g per well of pCGNGata4 or pCGNNkx2.5 were cotransfected using Lipofectamine 2000. The total amount of DNA transfected was adjusted to 6.5 \(\mu\)g per well by adding vector DNA. The cultures were incubated in serum-free DMEM for 12 h and in DMEM 10% FCS for further 18 h. The cells were lysed in HKMG buffer (30) and incubated with 5’ biotinylated double stranded DNA segments including recognition sequences for Gata4 (-19 to +41 nucleotide segment from the Ifi204 gene), or for Nkx2.5 (-98 to –49 nucleotide segment from the Ifi204 gene) at 4°C for 16 h. DNA-bound protein was pulled down by incubation with Streptavidin-agarose beads (Novagen) at 4°C for 1 h. The proteins were eluted with boiling SDS loading buffer, and the Gata4 and Nkx2.5 recovered were assayed by Western Blotting. For further details see (30).

Assay of the acceleration of the degradation of Id3 by p204 in vivo. BLK cells were plated on 10 cm tissue culture dishes. After a 24 h incubation, they were transfected using 2 \(\mu\)l Lipofectamine 2000 per \(\mu\)g DNA with 10 \(\mu\)g pCMV-Id3-Flag (encoding Id3-fused at its C-terminus to three Flag moieties), as indicated without or with 10 \(\mu\)g pCMV204 or 10 \(\mu\)g.
pCMV204NES. 24 h later the cultures were digested with Trypsin-EDTA, pooled, replated into 6 cm tissue culture dishes, and incubated for 24 h. At this time, 100μg/ml CHX was added, if so indicated, together with 20μM MG132. After the indicated times of incubation, the various cultures were lysed in TBS buffer (50mM TrisCl pH 7.4, 150mM NaCl, 1% Triton X100, 1mM Phenylmethylsulfonylfluoride, 1mM EDTA, 1μg/ml Leupeptine, 1μg/ml Aprotinin and 1μg/ml Pepstatin). The lysates were analyzed for Id3-Flag by Western Blotting with M2-Flag antibodies (Sigma). The bands were assayed using ImageQuant™ 5.2 software, and the data plotted using the SigmaPlot graphing program.

**Assay of the ubiquitin E1 activating enzyme dependent acceleration of Id3 degradation by p204 in vivo.** tsBN75 cells (28) carrying a thermosensitive E1 enzyme were cultured in 10cm dishes in 5% CO2 at 34° C and were transfected using 2μl Lipofectamine per μg DNA with 8 μg pCMV-Id3Flag (in which three Flag moieties were fused to the C terminus of Id3), and, as indicated, without or with 8 μg pCMV204, and without or with 8 μg pCMV-E1. After 24 h the cultures were digested, pooled and replated in 6 cm dishes and incubated at 40° C for 24 h. The cultures were then supplemented with 100 μM CHX and 20 μM MG132, harvested at the various times indicated and lysed. The lysates were analyzed for Id3-Flag as described in the previous section.

**Assay of the increase in the ubiquitination of ectopic Id3 by ectopic p204 in tsBN75 cells.**

The mammalian expression plasmids based on the pCMV vector and encoding the proteins specified in Figure 7 were transfected with Lipofectamin 2000 into tsBN75 cells. The plasmids used and the amounts of the plasmids transfected were the following: pCMV-HA-Ub (2μg, cultures 1-5), p3Xflag-CMV14-Id3 (2μg, cultures 1-4), pCMV204 ((2μg or 6μg, cultures 1 and 2, respectively), pCMV vector (2μg, culture 3, and 6μg, cultures 4 and 5). After incubation in 5% CO2 for 36h, 20μM MG132 was added and the cultures were incubated for 5h. After washing the cells they were lysed in RIPA buffer (50mM Tris-HCl pH7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EDTA, 1 mM PMSF, 1μg/ml each of Aprotinin, Leupeptin and Pepstatin, 1mM Na3VO4 and 1mM NaF) supplemented with 100μM NEM and 10μM ubiquitin aldehyde (Sigma), on ice for 30 min and centrifuged thereafter at 4° C and 14,000g for 15 min. 500μl aliquots (500μg protein) from the supernatant fractions were precleared with proteinG beads (Invitrogen) for 30 min and the residual solutions were incubated with proteinG beads conjugated with mouse antiFlag antibodies (Sigma) at 4° C overnight. After washing the beads 4 times with the supplemented RIPA buffer they were boiled in SDS loading buffer for 10 min. 30μl samples were analyzed by Western Blotting using 4-15 % SDS-PAGE and HA antibodies conjugated to HRP (12CA5, Roche).

**RESULTS**

**Ectopic Id3 inhibited the differentiation of P19 cells to myocytes as induced by DMSO. Ectopic p204 overcame this inhibition.**

As noted, the Id proteins inhibit skeletal muscle differentiation by binding the myogenic bHLH transcription factors and blocking their binding to DNA (18,19). We reported earlier that p204 can overcome this inhibition by the Id proteins (17). The fact that the level of p204 is even higher in mouse heart than in skeletal muscle (16) prompted us to explore whether the Id proteins also affect the differentiation of P19 cells to cardiac-type myocytes.

(INSERT FIGURE 1 HERE)

Fig. 1 reveals that (i) ectopic Id3 inhibited a characteristic of the differentiation to myocytes, i.e. the accumulation of MHC which is a component of the contractile machinery in myocytes induced in P19 cells by DMSO (cf. e and k), and (ii) ectopic p204 overcame this inhibition (cf. h and e). Ectopic p204 also strongly decreased the accumulation of ectopic Id3 (cf. d and g). As expected ectopic Id3 (tested in the absence of DMSO treatment) did not induce MHC (b). The findings (i) and (ii) prompted us to examine whether the expressions and/or the activities of the Gata4 and the Nkx2.5 transcription factors (31,32) are affected by the Id proteins and/or p204. Gata4 and Nkx2.5 are the earliest markers of precardiac cells in the course of cardiac differentiation (for a review see e.g. (33)).
Ectopic Id3 inhibited the expression of the Gata4, Nkx2.5 and MHC proteins in P19 cultures induced to differentiate to myocytes by DMSO.

The Western Blots in Fig. 2 compare the kinetics of expression of Gata4, Nkx2.5, MHC and (endogenous) Id3 proteins in control P19 cells induced to differentiate by DMSO (left panel), and the effect of ectopic Id3 on these kinetics (right panel). In the control culture Gata4 was detected on D4 and its level increased by D6. Both Nkx2.5 and MHC became detectable only on D6. The low level of endogenous Id3 in proliferating P19 cells (D0) slightly increased by D2, but thereafter, in the presence of DMSO, it strongly decreased by D4, and much further by D6. The presence of ectopic Id3 slightly decreased the levels of Gata4 on D4 and D6, strongly diminished the level of Nkx2.5 on D6, and decreased the level of MHC on D6 to a barely, if at all detectable level (right panel).

The apparent correlation of the decrease in the levels of MHC and Nkx2.5 with that of Gata4 are in accord with the findings that Gata4 lies upstream of Nkx2.5 in the regulatory cascade of cardiogenesis, and that Gata4 is among the transcription factors involved in the transcription of a and b MHC. It is also relevant that Gata4 and Nkx2.5 are mutual cofactors cross-regulating each others expression.

The decrease in the levels of expression of Gata4, Nkx2.5 and MHC proteins in the presence of ectopic Id3 prompted us to explore whether: (i) Id proteins bind Gata4 and/or Nkx2.5, and if so (ii) whether the Id proteins inhibit the transcriptional activities of Gata4 and Nkx2.5.

Interaction of the Id proteins with Gata4 and Nkx2.5.

The Western Blots in Figure 3A show a coimmunoprecipitation by Flag of ectopic Flag-tagged Id1, Id2 or Id3 with ectopic Gata4 (in (a)) and Nkx2.5 (in (b)) from lysates of 293 cells. As negative controls we established that rabbit IgG did not precipitate Gata4 or Nkx2.5 without or in the presence of Flag-Id2. These findings indicated the interaction of Gata4 and Nkx2.5 with each of the three Id proteins in vivo.

The GST-pull down assays in B revealed the binding of purified Id proteins (actually GST-Id1, GST-Id2 and GST-Id3) to purified Gata4 (actually MBP-Gata4) in vitro. As a negative control we established that the GST-Id proteins did not bind MBP, and GST did not bind MBP-Gata4.

The schematic structure of GST-Id2 with the locations of the N terminal, HLH and C terminal domains of Id2 is shown in C(b). The radioautograph (in C(a)) revealed that [35S] Gata4 could bind to both the HLH domain and the C terminal domain of Id2. Thus Gata4, a zinc finger protein, differs in its mode of binding to Id2 from the bHLH proteins e.g. MyoD. The latter bind primarily or only to the HLH domain of the Id proteins.

The schematic domain structure of Gata4 is shown in the top panel of D(b). The radioautographs revealing the interactions of various segments of Gata4 with Id2 and Id3 in a pull down assay are shown in D(a), and the interpretation of the relative strengths of the interactions with the segments is indicated in lines 1-13 of D(b). The results reveal some difference among the domains in Gata4 which bind to Id2 and Id3. Both segments of the N-domain i.e. 1-80, and 80-215 were needed for strong binding to Id2. The segment (241-269) did also bind Id2 but more weakly, at least if linked to more upstream or more downstream segments. The second segment of the N domain i.e. 80-215 was the primary site of binding to Id3. It should be kept in mind that these binding results may not perfectly reflect on the domains which bind to the Id proteins in complete Gata4. This is the case since the folding of isolated domains might be different from their folding when present in the complete protein.

The Id proteins inhibited the synergistic transactivation of reporter constructs by Gata4 and Nkx2.5 in consequence of inhibiting (i) the sequence-specific binding of Gata4 and Nkx2.5 to DNA, and (ii) the binding of Gata4 to Nkx2.5. p204 overcame the inhibition of the transactivation at least partially.

We tested (i) whether ectopic Id proteins inhibit the synergistic transactivation of reporter constructs including Gata4 and Nkx2.5 recognition sequences by Gata4 and Nkx2.5 in transfected cells, and if so, (ii) whether ectopic p204 can
overcome the inhibition at least partially. The experiments in Fig. 4A (a) involved the use of 10T1/2 fibroblasts and a reporter from the 5’ flanking region of the Ifi204 gene.

When equal amounts of Id1, Id2 or Id3 expression plasmids were transfected, into 10T1/2 cells the strongest inhibition of transactivation (65%) was exerted by Id3, and ectopic p204 decreased this inhibition to 23%. Thus in 10T1/2 cells in our conditions the inhibition of transactivation by ectopic Id3 was only partial, and ectopic p204 strongly decreased this inhibition. We expected that endogenous p204, if present, would contribute to the decrease of the inhibition. A Western Blot assay (in (b)) of a 10T1/2 cell lysate revealed a pronounced level of p204. We reported earlier that p204 expression in BLK cells (and in various other cell lines derived from C57BL/6 mice) was very low (37). Thus we expected that the endogenous p204 level in a BLK cell lysate should be much lower than that in a 10T1/2 cell lysate. This expectation was verified in the Western Blot in (b). In the experiment in (c) we used BLK fibroblasts and a reporter construct based on the cardiac ANF gene, whose expression is also transactivated by Gata4 and Nkx2.5 (38,39). The diagram in (c) showed (i) the strong synergy in the transactivation between Gata4 and Nkx2.5, and (ii) the dependence of the inhibition of the transactivation on the dosage of Id3. Moreover with the highest Id3 dosage 91% inhibition was noted, and this was diminished by ectopic p204 to 30%.

These results established that (i) Id proteins inhibited the synergistic transactivation of gene expression by Gata4 and Nkx2.5 in a dosage-dependent manner, and (ii) p204 overcame this inhibition at least partially. Synergistic transactivation of gene expression by Gata4 and Nkx2.5 requires that (i) one of the two transcription factors should bind to DNA in a sequence-specific manner, and (ii) the second transcription factor (which is not bound to DNA) should bind to the factor bound to DNA by protein-protein interaction (33,40).

Oligodeoxynucleotide assays combined with Western Blots (Fig.3B middle panels) and the diagrams (top panels) show the partial inhibitions of the sequence-specific binding to DNA of Gata4 (in (a)) and Nkx2.5 (in (b)) by Flag-Id1, Flag-Id2 and Flag-Id3, and the decrease of the inhibition by p204. When using equal levels of the three Id proteins (bottom panels) the binding of Gata4 was most strongly inhibited by Flag-Id2 (approx. 65%) and that of Nkx2.5 by Flag-Id1 (approx. 59%) (see the diagrams in the top panels). At the concentration tested p204 diminished the above inhibitions in the case of Gata4 (to approx. 22%), and in the case of Nkx2.5 it eliminated the inhibition.

Synergistic transactivation by Gata4 and Nkx2.5 involves the binding of the two transcription factors to one another (33,40). The Radioautographs in C (left panel) show the extent of the binding of [35S] Gata4 to MBP-Nkx2.5, and the dosage-dependent inhibition of this binding by Id1, Id2, or Id3. As also shown in the diagrams (in the right panel) Id2 inhibited the binding more than Id1, and Id3 even more than Id2. At the highest concentration of Id1, the inhibition was about 60%, of Id2 about 80% and of Id3 the inhibition was essentially complete. It was unexpected that the addition of various amounts of the p204 protein diminished these inhibitions at most only slightly (data not shown).

Thus in vivo p204 strongly diminished the Id3 dosage-dependent inhibition of the synergistic transactivation of gene expression by Gata4 and Nkx2.5 (Fig4A (c)). In vitro (i) p204 partially overcame the inhibition of the sequence-specific binding to DNA of Gata4 and Nkx2.5 (in B). However, (ii) p204 diminished the inhibition of the binding of Gata4 to Nkx2.5 in vitro if at all, only slightly (in C, and not shown).

Ectopic p204, but not p204ΔNES, accelerated the degradation of Id3 by proteasomes in BLK cells.

In a study concerning the role of p204 in skeletal muscle differentiation involving the use of C2C12 myoblasts (17) we reported that p204 promoted a decrease in the level of Id proteins. The experiment in Fig. 5A (INSERT FIGURE 5 HERE) involving BLK cells also revealed an ectopic p204 dosage-dependent decrease in the level of Id3. At the highest p204 level tested, Id3 became undetectable after 36h. As revealed by the RT-PCR assays (in Fig. 5B) ectopic p204 did however not effect the level of Id3 mRNA. These findings prompted us to assay whether p204 accelerates the
degradation of Id3 protein (actually we used Id3-Flag in which the Flag moiety was fused to the C terminus of the Id3 moiety) in BLK cells. The degradation of Id3 protein was reported to occur through the ubiquitin-proteasome pathway (41,42). The Western Blots in Fig. 5C and the diagram in Fig. 5D revealed that (i) in BLK cells in which protein synthesis was blocked by CHX ectopic p204 accelerated the degradation of Id3 (it decreased the t1/2 from 1.3h to 0.41h), and (ii) the degradation was inhibited by MG132, an agent blocking protein degradation by proteasomes. (iii) Remarkably, p204ΔNES did not accelerate the degradation of Id3, actually it slightly diminished this rate (it increased the t1/2 from 1.3h to 1.8h). It will be shown in a later section that this inability of p204ΔNES to accelerate the degradation of Id3 is not due to an inability of p204ΔNES to bind Id3 (see Fig. 9B).

**p204 accelerates the degradation of Id3 by the ubiquitin-proteasome pathway.**

We wanted to establish whether the acceleration by p204 of the degradation of Id3 by proteasomes was dependent on ubiquitination. For this propose we used (i) tsBN75 cells carrying a thermosensitive E1 ubiquitin activating enzyme which is known to be inactivated by incubation of the cells at 40°C (28), and (ii) as a positive control we transfected a culture of tsBN75 cells with an expression plasmid encoding wild type E1 which retains activity even if the cells are incubated at 40°C (43).

**(INSERT FIGURE  6 HERE)**

The results in Figure 6 revealed that p204 did not accelerate the degradation of Id3 (actually of Id3-Flag) in tsBN75 cells incubated at 40°C (Fig. 6A) (t1/2 of Id3 over 5h in the absence or presence of p204). p204 did however strongly accelerate the degradation of Id3 in tsBN75 cells carrying ectopic wild type E1 ubiquitin ligase, even if the culture was incubated at 40°C (Fig. 6B) (t1/2 of Id3 in the absence of p204 1.71h, t1/2 of Id3 in the presence of p204 0.47h).

Thus p204 clearly accelerated the degradation of Id3 by the ubiquitination-dependent proteasome pathway.

**p204 increased the ubiquitination of Id3 in a dosage dependent manner.**

**(INSERT FIGURE 7 HERE)**

To establish whether p204 can increase the ubiquitination of an Id protein in vivo we introduced into tsBN75 cells plasmids encoding Flag-tagged Id3 and HA-tagged ubiquitin without or together with various amounts of a plasmid encoding p204. After incubation to allow the expression of the ectopic proteins we added MG132, an inhibitor of proteasome activity, to allow the accumulation of polyubiquitinated Flag-tagged Id3. Culture lysates were prepared in the presence of ubiquitin-aldehyde, an inhibitor of deubiquitinating enzymes. The polyubiquitinated–Flag-tagged Id3 was immunoprecipitated with Flag, and the immunoprecipitate was analyzed by Western Blotting with antibodies to HA-ubiquitin. The Western Blot in Figure 7 reveals a p204 dosage dependent increase in the extent of Flag-Id3 polyubiquitination: The level of ubiquitination was low in the control reaction mixtures without p204 (3 and 4), higher in the reaction mixture supplemented with 1X p204 (1), and much higher in that supplemented with 3X p204 (2).

These results reveal that p204 strongly promoted the ubiquitination of Id3 in a dosage dependent manner. p204 is lacking regions homologous to known ubiquitin ligases or ubiquitin binding motifs. Thus it is likely that p204 promotes ubiquitination by one or several ubiquitin ligases by binding to the ligase(s) directly or in complex with other protein(s).

**Ectopic p204, but not p204ΔNES decreased the levels of endogenous Id proteins in proliferating P19 cells.**

**(INSERT FIGURE 8 HERE)**

As shown in the Western Blots in Fig. 8A (bottom panels) and the diagrams (top panels) (R.I.L. stands for Relative Id Level) transfection of pCMV204 (encoding p204) decreased the levels of endogenous Id1, Id2 and Id3 proteins in P19 cultures proliferating (in the absence of DMSO). By 36h after the transfection the ectopic p204 resulted in an approx. 65% decrease in the level of Id1, a 95% decrease in the level of Id2 and a 63% decrease in the level of Id3.

The Western Blots and diagrams in B reveal that ectopic p204ΔNES did however not decrease the levels of Id proteins. This lack of effect of p204ΔNES on the Id protein levels might be correlated with the inability of p204ΔNES to
induce the differentiation of P19 cells to cardiac myocytes (see Fig. 1C and also the Discussion section). The Western Blots and diagrams in C established (i) that the levels of endogenous Id1, Id2 and Id3 decreased by Day 8 of the differentiation process triggered by DMSO below 5% of their level in proliferating cell, and (ii) ectopic 204AS RNA strongly diminished or at least slowed down the decrease in Id protein levels. These findings are in accord with the inhibition of the differentiation of P19 cells as induced by DMSO by ectopic 204AS RNA (23).

**Ectopic p204, but not p204ΔNES, promoted the translocation of ectopic Flag-Id3 from the nucleus to the cytoplasm in differentiating P19 cells (Western Blot analysis of nuclear and cytoplasmic fractions from cell lysates).**

P19 cells stably transfected with pCMV-Flag-Id3 and, if so specified, with plasmids encoding p204, p204ΔNES, or the pCMV vector (Con) were incubated with DMSO and cultured in differentiation conditions (Fig. 9 A top panel). On Day 6 of the process of differentiation, MG132 was added to block the proteasomal degradation of Flag-Id3. After a further 2h incubation, nuclear and cytoplasmic fractions were prepared from the cell lysates and aliquots containing equal amounts of protein were tested by Western Blotting with antibodies to Flag (Fig. 9A top panel). The diagram (bottom panel) revealed that the ratio of the nuclear to cytoplasmic pFlagId3 was 1.55 in the control lysate (Con), 1.6 in the lysate from cells with ectopic p204ΔNES (+p204ΔNES) and approx. 0.65 in the lysate from cells with ectopic p204 (+p204). Control experiments established that (i) the levels of expression of p204 and p204ΔNES were similar in the two cultures (as shown in the Western Blot in A middle left panel). Furthermore (ii) as shown in the Western Blot in B Flag coimmunoprecipitated similar amounts of Id3 with Flag-p204 and with Flag-p204ΔNES. This demonstrated that the lack of the NES did not alter the binding of Flag-p204 to Id3. Consequently, the differences in the ratios of nuclear/cytoplasmic Flag-Id3 in the diagram in A (bottom panel) indicated that p204 promoted the translocation of Flag-Id3 from the nucleus to the cytoplasm, and this promotion by p204 depended on the presence of the NES.

Induction of endogenous p204 resulted in the translocation of the bulk of GFP-tagged Id3 from the nucleus to the cytoplasm in C2C12 myoblasts differentiating to myotubes (Immunofluorescence microscopy).

The experiments in Fig. 9C involved the use of a C2C12 cell line (ind.p204 (C2C12)) in which the expression of p204 could be induced (about 3 fold) by Muristerone, and a control C2C12 line (Con (C2C12)), in which Muristerone did not affect the level of p204 (see the Western Blots in the bottom left panel) (16). The immunofluorescence microscopic pictures in the top panel show that in cells of the differentiating Con and ind.p204 lines tested in the absence of Muristerone (—) the ectopic GFP-Id3 was distributed between the nucleus and the cytoplasm, with more in the nucleus than in the cytoplasm. The addition of Muristerone (+) to both lines had no effect on the localization of GFP-Id3 in the Con (C2C12) line whereas in the Ind.p204 (C2C12) line it resulted in the translocation of the bulk of GFP-Id3 to the cytoplasm. The control experiments ((GFP(Con) in the bottom right panel of C) showed that GFP is distributed between the nucleus and the cytoplasm without and after exposure to Muristerone. Thus the results (in A) based on the fractionation of the lysates from differentiating P19 cells into nuclear and cytoplasmic fractions, and (in C) based on fluorescence microscopy of differentiating C2C12 skeletal muscle cells both indicated that p204 (but not p204ΔNES) could trigger the translocation of Id3 from the nucleus to the cytoplasm.

Thus p204 was able to promote at least three processes that p204ΔNES could not: p204 (i) triggered the differentiation of P19 cells to beating cardiac myocytes, (ii) it accelerated the degradation of Id3 protein by the ubiquitin-proteasome pathway, and (iii) it boosted the translocation of Id3 protein from the nucleus to the cytoplasm in differentiating P19 cells.

**DISCUSSION**

Most of our study on the involvement of p204 in cardiac myocyte differentiation was performed with P19 cells. The fact that these differentiated into beating cardiac myocyte type cells greatly increased the confidence in the relevance of P19 cells as a model system. It has
been widely used during the last twenty three years and it provided much insight into the process of the differentiation including the transcription factors, and the upstream signaling pathways involved (44).

In the case of skeletal muscle differentiation as studied in the C2C12 myoblast system, we determined that one of the functions of p204 in the process is to overcome the inhibition of the differentiation by the Id proteins (17). The present studies concerning the differentiation of P19 cells to cardiac myocytes reached the same conclusion concerning the function of p204. However, in the case of skeletal muscle differentiation, the targets of inhibition by Id were known to be the bHLH transcription factors (e.g. MyoD, myogenin, E12 and E47) (18,19,45). Other non bHLH proteins were also found to be targets for at least some of the Id proteins. These targets include ETS domain transcription factors, Pax-2/,-5/,-8 subfamily proteins, ADD/SREBP-1c factor and pRb protein (46-50).

In the case of cardiac myocyte differentiation no proteins were known to be targets of Id proteins. We established in this study that, similarly to the case in differentiating C2C12 skeletal muscle myoblasts, ectopic Id proteins inhibited P19 differentiation and p204 overcame this inhibition. In the developing mouse heart Id1, Id2 and Id3 were detected in the epicardium and endocardium (51).

We determined that the levels of the Id1, Id2 and Id3 proteins were the highest in proliferating P19 cells, and during the differentiation to cardiac myocytes these levels gradually decreased becoming undetectable. Triggering the differentiation by either DMSO or p204 resulted in the expression among other proteins of Gata4 and Nkx2.5, crucial transcription factors for the process. We determined that both Gata4 ( a zinc-finger protein)(31), and Nkx2.5 ( a homeobox protein) (52) were novel types of targets of the Id proteins, and we determined the regions in the Id proteins and in Gata4 which bound each other. The ectopic Id proteins inhibited the synergistic transactivation by Gata4, Nkx2.5 and Tbx5 of the expression of cardiac reporter genes (based on the Ifi204 and the ANF genes), and p204 (which bound the Id proteins) overcame this inhibition in vivo.

The Id proteins inhibited two steps involved in the synergistic transactivation of gene expression by Gata4 and Nkx2.5: (i) the sequence specific binding of each protein to DNA, and (ii) the binding of Gata4 to Nkx2.5, that is required for the synergy. In vitro p204 partially overcame the inhibition of step (i), but it diminished the inhibition of step (ii) if at all, only very weakly. In vivo however ectopic p204 strongly decreased the level of endogenous and also of ectopic Id proteins without altering the levels of Id mRNAs in various cells including P19. This decrease in Id level strongly contributed to the inhibition of Id activity by p204. p204 decreased the level of the Id proteins by accelerating their degradation by the ubiquitin-proteasome pathway. In line with this finding, 204 antisense RNA (which inhibited the expression of p204) decreased the degradation of endogenous Id proteins in differentiating P19 cells.

It was remarkable that the acceleration by p204 of the degradation of Id proteins by the ubiquitin- proteasome pathway strictly depended on the presence of the NES in p204, and this was the case in spite of it that p204NES bound the Id proteins as well as p204 did. This need for the NES in p204 for the acceleration of Id protein degradation was in line with the finding that NES was also required for p204 (i) to trigger P19 differentiation, (ii) to induce Gata4, Nkx2.5 and MHC in P19 cells, (iii) to become translocated from the nucleus to the cytoplasm during the differentiation (23), and finally (iv) to promote the translocation of Id proteins from the nucleus to the cytoplasm during P19 cell differentiation. The interaction of p204 with the Tpr protein, a component of the nuclear pore complex, is likely to be involved in the translocation (53,54). One of the ways by which p204 accelerated the degradation of Id proteins by the ubiquitin-proteasome pathway was the acceleration by p204 of the ubiquitination of Id proteins (Figure 7). As noted, the sequence of p204 does not reveal the presence of domains with ubiquitin ligase activity. Thus, the identity of the ubiquitin ligase(s), whose activity is increased by p204 and which degrades the Id proteins remains to be identified.

As indicated earlier (i) Gata4, Nkx2.5 (and Tbx5) synergistically transactivated the expression of p204 (23), and (ii) the accumulated p204 decreased the inhibition of
Gata4 and Nkx2.5 activity by the Id proteins, and thereby increased the transactivation of gene expression (including the expression of p204) by Gata4 and Nkx2.5. Furthermore (iii) Gata4 and Nkx2.5 were shown to promote the expression of each other (55,56). These facts indicated the existence of a positive feedback loop linking the functioning of Gata4 and Nkx2.5 to that of p204 (Fig. 10). This positive feedback loop may account for the apparently paradoxical fact that ectopic p204 triggered the expression of Gata4 and Nkx2.5, and at the same Gata4 and Nkx2.5 synergistically transactivated the expression of p204. We reported earlier that a similar positive feedback loop including p204 and Id proteins functioned in skeletal muscle C2C12 myoblast differentiation, however in the myoblast differentiation loop, MyoD and/or myogenin substituted for Gata4 and Nkx2.5 (17).

Together with the discovery that the Id proteins are degraded by the ubiquitin-proteasome pathway, it was reported that the degradation of Id3 was slowed down when it was bound to a target bHLH protein (MyoD) (41,57). In accord with this finding, we observed that ectopic Gata4, another target of Id proteins, also retarded the degradation of Id3 by the ubiquitin-proteasome pathway in proliferating P19 cells, and ectopic p204 diminished this retardation (data not shown). The enhancement by p204 of the translocation of the Id proteins from the nucleus to the cytoplasm may facilitate the differentiation of P19 cells in various ways: (i) by removing from the nucleus the inhibitory Id proteins, it is likely to increase the synergistic transactivation of cardiac genes by Gata4, Nkx2.5, and Tbx5. The validity of this assumption was supported by the following findings (58) ectopic Id3 (which is lacking an NES) was uniformly distributed between the nucleus and the cytoplasm in NIH3T3 cells. It strongly inhibited the transcriptional activity of target bHLH proteins (MyoD, E47). The linkage of a NES domain to the Id3 resulted in a fusion protein which, upon transfection, was predominantly cytoplasmic. This inhibited the activity of the bHLH proteins to a much lesser extent. (ii) Proteasomes were found to exist in both the nucleus and the cytoplasm (59). By translocating the Id proteins from the nucleus to the cytoplasm, they may be separated from their target proteins (e.g., Gata4) which can retard their degradation. In consequence of this, the degradation of the Id proteins in the cytoplasm via the ubiquitin-proteasome pathway can be accelerated. A need for the translocation of particular proteins from the nucleus to the cytoplasm to enable or accelerate their degradation by proteasomes was described in numerous publications (60-62). It should be noted however that in the case of neural progenitor cells the OLIG1 and OLIG2 bHLH transcription factors are predominantly nuclear, whereas after exposure of the cells to BMP4, which induces Id proteins and inhibits oligodendrial differentiation, OLIG1 and OLIG2 in complex with Id4 and Id2 (but not Id1 or Id3) are translocated to the cytoplasm (63). It is probable that the degradation of the Id proteins upon their translocation from the nucleus to the cytoplasm might be enabled or accelerated in consequence also of effects other than the separation from their targets (e.g. the localization of a factor required for the degradation primarily in the cytoplasm).

These studies reporting the involvement of p204 in P19 stem cell differentiation to cardiac type myocytes also raised several new problems that remain to be explored. Thus, p204 was translocated from the nucleus to the cytoplasm during the differentiation of both C2C12 myoblasts to myotubes (16) and P19 cells to cardiac type myocytes (this study). This translocation of p204 in turn promoted the translocation of Id proteins, and this was a prerequisite for their accelerated degradation. p204 was found to be phosphorylated in the cytoplasm, whereas in the nucleus it was not ((16) and Ding, unpublished). These finding suggest that the phosphorylation of p204 was required for its translocation. The identity of the kinase(s) involved, and of the amino acid(s) phosphorylated in p204 remain to be established.

As shown in this study, p204 also accelerated the degradation of Id proteins by increasing their ubiquitination. It remains to be determined whether p204 also affects any of the various steps (64) occurring between polyubiquitination and proteasomal degradation.

p204 also overcame (i.e., “antagonized”), at least partially, the inhibition by the Id proteins of the sequence-specific binding to DNA of the
cardiac Gata4 and Nkx2.5 (Figure 4B), as well as of the skeletal muscle MyoD and E47 transcription factors (17). These findings may indicate that this “antagonistic” activity of p204 is one of the various mechanisms by which p204 promotes skeletal and cardiac muscle differentiation. However, as yet, we have no proof in either case either of the requirement, or of the dispensability, of this “antagonistic” activity of p204 for overcoming the inhibition of differentiation by the Id proteins in vivo. Thus, at present, this “antagonistic” activity of p204 should be considered a potential participant in the promotion of differentiation by p204 whose participation remains to be established. However, p204 promotes the differentiation (besides those of skeletal and cardiac muscles) also e.g. of osteoblasts and chondrocytes (15). Thus there exist various other processes in which this “antagonistic” activity of p204 might turn out to be essential.

Various other known activities of p204 were not considered in this study. These include the inhibition of ribosomal RNA synthesis, the binding of the pocket proteins pRb, p107, and p130, and the retardation of cell proliferation (10, 12, and 14). It remains to be established whether any of the above activities is required for promoting the differentiation of P19 cells to cardiac type myocytes.

Finally, it should be noted that, according to a recent report, Id1, Id2, and Id3 were not detected in the myocardium of day 10.5 - 16.5 mouse embryos, (although Id1, Id2 and Id3 were found to be required for mouse heart differentiation) (51)Footnote 2). Proliferating P19 embryonal carcinoma stem cells, whose differentiation in vitro into beating cardiac myocyte type cells we have studied, expressed Id1, Id 2, and Id3. However the P19-derived, differentiated myocyte-type cells did not contain detectable levels of Id proteins. P19 cells are a type of pluripotent embryonal stem cells which, if injected into early mouse embryos, are capable of contributing to a variety of normal tissues (65). It remains to be seen whether the embryonal myocardium in which no Id proteins were detected contained, in addition to differentiated cardiac myocytes which lacked Id proteins, myocyte progenitors which were further differentiated than stem cells (possibly cardiac muscle lineage?) and have lost their Id proteins as well as their pluripotency. Until recently, all myocardial cells were thought to originate in the primary heart tube. Recently, a second population of myocardial precursors was discovered anterior to the heart tube (anterior heart field) (66) This population is the source, among others, of the myocardial cells of the embryonic right ventricle. It remains to be seen whether the myocardial progenitor cells in this population express Id proteins.

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REFERENCES


**FOOTNOTES**

Footnote 1: The abbreviations used are: 204AS, 204 antisense; a protein (e.g. a Gata4), antiserum to the protein in question (e.g. to Gata4); ANF, atrial natriuretic factor; bHLH, basic region-helix-loop-helix; CHX, cycloheximide; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethysulfoxide; GST, gluthathione transferase; Id, inhibitor of differentiation; MHC, myosin heavy chain; NES, Nuclear Export Signal; p204ΔNES, p204 lacking the NES; p204MNES, p204 with a mutated NES; R.I.L., Relative Id Level; R.L.A., Relative Luciferase Activity; TR, Texas Red.

Footnote 2: Interestingly, the same study(51) also described that, in Id knockout embryos, among others, the development of the myocardium was impaired, and, furthermore, administration of insulin-like growth factor 1, and WNT5, two secreted factors, could partially correct the cardiac phenotype.
FIGURE LEGENDS

Figure 1. Ectopic Id3 inhibited the differentiation of P19 cells to myocytes as induced by DMSO. Ectopic p204 overcame this inhibition by Id3. Immunofluorescence Microscopy.

P19 cell lines stably expressing ectopic Id3 and control cell lines were generated by transfection of the pcDNA3-Id3 expression plasmid, or the pcDNA3 vector (serving as control), respectively, and selection with G418. Cultures of the cell line expressing ectopic Id3 were transfected with the pCMV-204 plasmid or the pcDNA3 vector. Three cultures from each of the resulting three types of cell lines (designated as Id3, Id3+p204 and Con) were incubated in tissue culture dishes for 2 days, transferred to petri dishes in differentiation medium with DMSO for 4 days, and, thereafter, shifted to tissue culture dishes for 3-4 days (Id3+DMSO, Id3+p204+DMSO, Con+DMSO). Three further cultures from the Id3 type were incubated as above except without DMSO (Id3). The cultures were examined by immunofluorescence microscopy using Id3 (a, d, g, j) or MF20 (MHC) antibodies (b, e, h, k), and visualized with FITC-labeled or Texas-red-labeled secondary antibodies. The nuclei were stained with DAPI (c, f, i, l). The bars correspond to 10 µm. For further details, see Experimental Procedures.

Figure 2. Ectopic Id3 inhibits the expression of the cardiac Gata4, Nkx2.5 and MHC proteins in P19 cells induced to differentiate by DMSO. Western Blotting.

Control (Con) P19 cultures stably transfected with the pCMV vector (left panels), and experimental P19 cultures transfected with pCMV-Id3 (right panels). Both types of cultures were induced to differentiate to cardiac myocytes by incubation with 0.8% DMSO. Cell lysates were prepared from both types of cultures on the days (D0-D6) indicated. The expressions of Id3, Gata4, Nkx2.5 and MHC proteins, and as an internal control of Tubulin protein were determined in aliquots from the lysates containing 80 µg total protein by Western Blotting using the appropriate antibodies. For further details see Experimental Procedures.

Figure 3. The Id1, Id2, and Id3 proteins could be coimmunoprecipitated with Gata4 or Nkx2.5 from the cell lysate. Binding of Gata4 to the Id proteins in vitro. Identification of the Gata4 and Id2 domains involved in the interaction.

A. Coimmunoprecipitation of ectopic Gata4 or Nkx2.5 with ectopic Id1, Id2, or Id3 from cell lysates. 293 cells were co-transfected with pCGN-Gata4 (in (a)) or pCGN-Nkx2.5 (in (b)), as well as with Flag-Id1, Flag-Id2, or Flag-Id3 expression plasmids. After a 40 h incubation, the various cultures were lysed, the lysates were immunoprecipitated with Flag antibodies, and, as a negative control, with rabbit IgG. The immunoprecipitates were assayed by Western Blotting in (a) first with Flag antibodies, in (b) first with Nkx2.5 (top panels). Thereafter, the membranes were stripped, and Western Blotting was performed, using Flag antibodies to assay the Id proteins (bottom panels). Aliquots from the input lysates (Input) were similarly examined by Western Blotting.

B. Binding of Gata4 to the Id proteins in vitro. Binding of purified Gata4 protein (actually MBP-Gata4) to purified Id proteins (actually GST-Id1, GST-Id2, and GST-Id3) in vitro was tested. MBP-Gata4 or as a control MBP were incubated with GST beads loaded with GST-Id1, GST-Id2, GST-Id3 or as a control GST . The MBP-Gata4 or MBP proteins retained on the various GST-Id beads and on GST beads were assayed by Western Blotting using rabbit MBP.

C. Identification of the Id2 domains binding Gata4. Three segments from Id2 (schematically shown in (b)) were fused to GST. The various GST-Id2 segment fusion were bound to GST-agarose, and incubated with [35S] Gata4 which had been translated in vitro. The GST-Id2 segments with the bound [35S] Gata4 were examined by SDS-PAGE and autoradiography (see (a)).

D. Identification of the Gata4 domains binding Id2 and Id3. Thirteen segments from Gata4 (schematically shown in (b)) were fused to MBP. The various MBP-Gata4 segment fusion proteins were bound to amylose beads and incubated with [35S] Id2 or with [35S] Id3 which had been translated in vitro. The MBP-Gata4 segments with the bound [35S] Id2 or [35S] Id3 were examined by SDS-PAGE and
Figure 4. The Id proteins inhibited the synergistic transactivation of reporter constructs by Gata4 and Nkx2.5 in consequence of inhibiting (i) the sequence-specific bindings of Gata4 and Nkx2.5 to DNA and, (ii) the binding of Gata4 to Nkx2.5. p204 partially overcame the inhibition of the synergistic transactivation by the Id3 protein.

A. Ectopic Id1, Id2, and Id3 inhibited the synergistic transactivation by Gata4 and Nkx2.5 of reporter constructs driving luciferase expression in cultured cells. Ectopic p204 partially overcame the inhibition. (a) Id1, Id2, or Id3 inhibited the transactivation of a reporter construct based on the Ifi204 gene in 10T1/2 cells. p204 decreased the inhibition (luciferase assays). 10T1/2 cultures were transfected with a reporter construct driven by a regulatory segment from the Ifi204 gene and encoding luciferase, an internal control plasmid pSVGal, together, if so indicated, with expression plasmids encoding Gata4, Nkx2.5, p204, and different amounts of Id3. 36 h after transfection, the cultures were lysed, β-galactosidase and luciferase assays were performed and the Id3 dosage-dependent extent of the inhibition of the synergistic activation of luciferase by Gata4 and Nkx2.5, as well as the partial release of this inhibition by p204 and the standard deviations are indicated. For further details, see the Legend to A(a).

B. Flag-Id1, Flag-Id2, and Flag-Id3 inhibited the sequence-specific binding of Gata4 (in (a)), and Nkx2.5 (in (b)) to DNA. p204 decreased the inhibition (biotinylated oligodeoxynucleotide precipitation assay). 10T1/2 cells were transfected with expression plasmids encoding the proteins specified, i.e., pCMV-Flag-Id1, and/or pCMV-Flag-Id2, pCMV-Flag-Id3, pCMV-p204, as well as pCGN-Gata4 (in (a)), or pCGN-Nkx2.5 (in (b)). After a 30 h incubation, the cultures were lysed, and aliquots from the precleared lysates were incubated with a biotinylated double-stranded DNA oligodeoxynucleotides including recognition sequences for Gata4 (in (a)) or for Nkx2.5 (in (b)). After incubation the reaction mixtures were supplemented with streptavidin-agarose beads to bind the biotinylated oligodeoxynucleotides with the bound proteins, and the incubations were continued. After washing the beads, they were eluted, and the eluates were assayed by Western Blotting with a Gata4 (in (a)), or a Nkx2.5 (in (b)) (middle panels). The lysates were also tested for the expressions of Flag-Id1, Flag-Id2, Flag-Id3, as well as Gata4 (in (a)) and Nkx2.5 (in (b)) by Western Blotting (bottom panels). The blots in the middle panels were scanned and graphed using an Excel graph program (top panels).

C. (left panel) Id1, Id2, or Id3 inhibited the binding of [35S] Gata4 to immobilized Nkx2.5 in a dosage-dependent manner. [35S] Gata4 and Id proteins were obtained by in vitro translation, and were incubated with MBP-Nkx2.5 fusion protein attached to amylose beads. [35S] Gata4 bound to the beads was released and assayed by SDS-PAGE and autoradiography. (right panel) Diagram of the dosage-dependent inhibition of the binding of
[35S] Gata4 to MBP-Nkx2.5 by Id1, Id2, and Id3. For further details see Experimental Procedures.

Figure 5. Ectopic p204 decreased the level of ectopic Id3 in BLK cells by accelerating the degradation of Id3 by proteasomes.
A. Ectopic p204 decreased the levels of ectopic Id3 protein in BLK cells in a dosage-dependent manner. (Left panel) BLK cells in 6 cm dishes were transfected with 2 μg pCMV-Id3 and, if so indicated, with the amount of pCMV204 specified. After a 36 h incubation, the cells were lysed in TBS buffer and the levels of p204, Id3 and, as an internal control, tubulin, were determined by Western Blotting. (Right panel) Diagram of the p204 dosage-dependent decrease in the Id3 level in BLK cells as shown in the left panel.

B. p204 did not decrease the level of Id3 mRNA in BLK cells (RT-PCR). BLK cultures in 6 cm dishes were transfected with 2 μg pCMV-Id3 and either 4 μg pCMV204 or, as a control, with 4 μg pCMV vector. 36 h later, total RNA was isolated. The indicated amounts of total RNA samples were assayed for Id3 mRNA by RT-PCR, gel electrophoresis and visualization with Ethidium Bromide.

C. p204, but no p204ΔNES, accelerated the degradation of ectopic Id3-Flag by proteasomes in BLK cells. BLK cells were transfected as indicated with pCMV-Id3-Flag (a plasmid encoding Id3 fused at its C-terminus to 3 Flag moieties), if so indicated, together pCMV204 or pCMV204ΔNES. After 24 h, the cultures were digested with trypsin-EDTA, pooled, replated, and incubated for further 24 h. Thereafter (0 h), each culture was supplemented with CHX, and, if so indicated, with MG132. The various cultures were harvested and lysed after the indicated times of incubation (0.5 h to 4 h), and analyzed for Id3-Flag by Western Blotting, using Flag.

D. The protein bands in C. were assayed and graphed. The rate of degradation is expressed as t1/2, the time required for the degradation of 50% of Id3-Flag. The number of pixels at t=0 was taken as 100%. The log 10 of the % of pixels was plotted vs. time for each time point. For further details, see Experimental Procedures.

Figure 6. p204 accelerated the degradation of Id3 by proteasomes: dependence of the acceleration on ubiquitin activating enzyme (E1).

tsBN75 cells carry a thermosensitive E1 which is inactivated at 40º C. Transfection of a plasmid encoding wild type E1 (pCMV-E1) eliminates the thermosensitivity. tsBN75 cells cultured at 34ºC were transfected with pCMV-Id3Flag (encoding Id3 fused at its C terminus to 3 Flag moieties), and, if so indicated, pCMV204 (in A), or were transfected with pCMV-Id3Flag, pCMV-E1, and, if so indicated, with pCMV204 (in B). After 24 h, the cultures were digested with Trypsin-EDTA, pooled, replated, and incubated at 40º C for 24 h. At this time, the cultures were supplemented with CHX and were harvested and lysed after incubation at 40º C for the various times specified. The levels of Id3Flag were assayed by Western Blotting. The time courses of the degradation of Id3Flag were graphed as described in the legend to Fig. 5. For further details see Experimental Procedures.

Figure 7. p204 dosage-dependent increase in the ubiquitination of Id3 in transfected tsBN75 cells.

The mammalian expression plasmids encoding HA-Ub and Id3-F proteins were transfected into the cultures specified. Cultures 1 and 2 (p204) were also transfected with pCMV204 (2 μg or 6 μg respectively), cultures 3 and 4 (Con) and 5 were also transfected with pCMV vector (2 μg, 6 μg or 6 μg respectively). After incubation at 33 º C for 36 h the cultures were supplemented with MG132 and incubated for further 5 h. The culture lysates (prepared in a lysis buffer including ubiquitin-aldehyde an inhibitor of deubiquitination) were then immunoprecipitated with antibodies to Flag and the washed immunoprecipitates were analyzed by Western Blotting with antibodies to HA. For further details see Experimental Procedures.

Figure 8. Ectopic p204, but not p204ΔNES decreased the levels of endogenous Id1, Id2, and Id3 in proliferating P19 cells. Ectopic 204AS RNA inhibited the decrease in the levels of endogenous Id1, Id2 and Id3 proteins during the differentiation of P19 cells as induced by DMSO.
A. Ectopic p204 strongly decreased the levels of Id1, Id2, and Id3 in proliferating P19 cells, whereas B. Ectopic pCMV204ΔNES did not, it caused a very slight increase in the levels of these Id proteins (Western Blotting). P19 cells in 10 cm dishes were transfected with 5μg pCMV vector (Con) or 5μg pCMV-204 (in A) or pCMV204ΔNES (in B). After a 36 h incubation, the cells were harvested, lysed in lysis buffer, and the levels of the Id1, Id2, and Id3 proteins were determined by Western Blotting. In the graphs, the levels of the three Id proteins in the presence of ectopic p204 or p204ΔNES were compared to their levels in control cells (Con) which were taken as 1. RIL stands for Relative Id Level. As loading controls, the levels of Tubulin were determined.

C. The levels of endogenous Id1, Id2, and Id3 proteins decreased during the differentiation of P19 cells as induced by DMSO. Ectopic 204ASRNA retarded the decrease in the levels of the Id proteins (Western Blotting). P19 cells in 10 cm dishes were transfected with 5μg pCMV204 (p204) or 5μg pCMV204ΔNES (p204ΔNES) or with 5μg pCMV vector (Con) as well as with 5μg pCMVFlag-Id3 (in which the Flag-tag was attached to the N-terminus of Id3 to slow down the rate of ubiquitination) (67) were incubated with 0.8% DMSO and cultured in differentiation conditions. On D6, the cultures were supplemented with 20μM MG132 (to inhibit the digestion of Flag-Id3 by proteasomes). After a 2 h incubation, cells were harvested, pelleted, suspended in buffer, washed, and lysed in a Dounce homogenizer, and the nuclei were pelleted, and nuclear and cytoplasmic extracts were prepared following a published procedures (68). 60μg protein samples from each of the two extracts were analyzed by Western Blotting with anti-Flag M2 monoclonal antibodies. As loading controls, the levels of LaminA (a nuclear protein), and Tubulin (a cytoplasmic protein) were assayed by Western Blotting. The bottom graph shows the ratios of the levels of nuclear and cytoplasmic Flag-Id3. The diagram on the left of the middle panel shows a comparison of the levels of expression of ectopic p204 and p204ΔNES by Western Blotting.

B. p204 and p204ΔNES bind Id3 protein to a similar extent (pulldown assay and Western Blotting). To produce Flag-p204 and Flag-p204ΔNES, Cos cells in 6cm dishes were transfected with 6μg pCMVFlag-p204 or 6μg pCMVFlag-p204ΔNES, respectively, incubated for 24-30 h and lysed in lysis buffer. A/G beads with bound M2 antiFlag-monoclonal antibodies were loaded with equal amounts of Flag-p204 or Flag-p204ΔNES from the appropriate cell lysates. The loaded beads were incubated with [35S] labeled Id3 protein also obtained by in vitro translation. After washing the beads, the Id3 retained was eluted and assayed by gel electrophoresis and radioautography. As negative controls, A/G beads loaded with mouse IgG proteins were similarly incubated with [35S] labeled Id3, washed, eluted, and assayed by gel electrophoresis and radioautography.

C. The induction of p204 expression in differentiating C2C12 skeletal muscle myoblasts resulted in the translocation of a large portion of GFP-Id3 from the nucleus to the cytoplasm. Immunofluorescence microscopy. The ind.p204

Figure 9. Ectopic p204 but not p204ΔNES promoted the translocation of Id3 from the nucleus to the cytoplasm in P19 cells differentiating to myocytes.

A. Assay by fractionation of the cell lysates to nuclear and cytoplasmic components. Western Blotting. P19 cells in 10 cm dishes stably transfected with 5μg pCMV204 (p204) or 5μg pCMV204ΔNES (p204ΔNES) or with 5μg pCMV vector (Con) as well as with 5μg pCMVFlag-Id3 (in which the Flag-tag was attached to the N-terminus of Id3 to slow down the rate of ubiquitination) (67) were incubated with 0.8% DMSO and cultured in differentiation conditions. On D6, the cultures were supplemented with 20μM MG132 (to inhibit the digestion of Flag-Id3 by proteasomes). After a 2 h incubation, cells were harvested, pelleted, suspended in buffer, washed, and lysed in a Dounce homogenizer, and the nuclei were pelleted, and nuclear and cytoplasmic extracts were prepared following a published procedures (68). 60μg protein samples from each of the two extracts were analyzed by Western Blotting with anti-Flag M2 monoclonal antibodies. As loading controls, the levels of LaminA (a nuclear protein), and Tubulin (a cytoplasmic protein) were assayed by Western Blotting. The bottom graph shows the ratios of the levels of nuclear and cytoplasmic Flag-Id3. The diagram on the left of the middle panel shows a comparison of the levels of expression of ectopic p204 and p204ΔNES by Western Blotting.

B. p204 and p204ΔNES bind Id3 protein to a similar extent (pulldown assay and Western Blotting). To produce Flag-p204 and Flag-p204ΔNES, Cos cells in 6cm dishes were transfected with 6μg pCMVFlag-p204 or 6μg pCMVFlag-p204ΔNES, respectively, incubated for 24-30 h and lysed in lysis buffer. A/G beads with bound M2 antiFlag-monoclonal antibodies were loaded with equal amounts of Flag-p204 or Flag-p204ΔNES from the appropriate cell lysates. The loaded beads were incubated with [35S] labeled Id3 protein also obtained by in vitro translation. After washing the beads, the Id3 retained was eluted and assayed by gel electrophoresis and radioautography. As negative controls, A/G beads loaded with mouse IgG proteins were similarly incubated with [35S] labeled Id3, washed, eluted, and assayed by gel electrophoresis and radioautography.

C. The induction of p204 expression in differentiating C2C12 skeletal muscle myoblasts resulted in the translocation of a large portion of GFP-Id3 from the nucleus to the cytoplasm. Immunofluorescence microscopy. The ind.p204
(C2C12) cell line carried constructs allowing the induction of the expression of p204 by Muristerone. The level of p204 in the control line (Con (C2C12)) was unaffected by Muristerone (Western Blotting bottom left panel). Two 6 cm dishes of proliferating Con (C2C12) cells and two 6 cm dishes of proliferating ind.p204 (C2C12) cells in DMEM, 10% FBS were transfected with 3μg/dish pCMVGFP-Id3. 12h later all four dishes were shifted to skeletal muscle differentiation medium: DMEM, 1% horse serum. 2.5μM Muristerone was added to one dish from both types of cells (+) and the incubation was continued for 36-48h. The subcellular location of GFP-Id3 in the four dishes was examined by fluorescence microscopy (upper panels). Two further 6cm dishes of proliferating ind.p204 (C2C12) cells were transfected with 3μg pCMV-GFP. 12h later both dishes were shifted to differentiation medium. 2.5μM Muristerone was added to one of the dishes (+) and the incubation was continued for 36-48h. The subcellular locations of GFP was examined by fluorescence microscopy (bottom right panel). For further details, see Experimental Procedures.

Figure 10. Positive regulatory feedback loop among the Id1, Id2, Id3, Gata4, Nkx2.5, and p204 proteins functioning during the differentiation of P19 stem cells to beating cardiac myocytes. Multiple roles of the various proteins in the process.

\( a \) The Id proteins bind the Gata4 and Nkx2.5 transcription factors and inhibit their synergistic transactivation of the expression of p204 and other cardiac proteins (e.g., ANF) by inhibiting the binding of Gata4 and Nkx2.5 (i) to DNA and (ii) to each other.

\( b \) Gata4 and Nkx2.5 synergistically transactivate the expression of p204.

\( c \) The inhibition of the activity of Gata4 and Nkx2.5 by the Id proteins is overcome by p204 by (i) binding and sequestering the Id proteins, and, thus, inhibiting their binding of Gata4 and Nkx2.5, and (ii) by promoting the translocation of the Id proteins from the nucleus to the cytoplasm and, thereby, (a) separating the Id proteins from the nuclear Gata4 and Nkx2.5 and (b) accelerating the ubiquitination and degradation of the Id proteins by proteasomes. The promotion of the translocation to the cytoplasm and of the degradation of Id proteins by p204 depend on the NES (Nuclear Export Signal) in p204.

\( d \) By overcoming the inhibition of Gata4 and Nkx2.5 activity by the Id proteins, p204 also promotes the mutual enhancement of the expression by Gata4 and Nkx2.5. For further details, see the text.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

A

P19 cells (D6,+DMSO)

<table>
<thead>
<tr>
<th></th>
<th>Nuclear extract</th>
<th>Cytoplasmic extract</th>
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<tr>
<td>p204</td>
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</tr>
<tr>
<td>ΔNES</td>
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<td>+</td>
</tr>
<tr>
<td>Con</td>
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MG132

- αFlag(Id3)
- αTubulin
- αLamin A

Rate of nuclear/cytoplasmic Flag:id3

B

IP αFlag

mouse IgG

Input Flag:p204 Flag:p204ΔNES Flag:p204 Flag:p204ΔNES

 [$^{35}$S]Id3

C

Muristerone

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<th>Con(C2C12)</th>
<th>ind.p204(C2C12)</th>
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Muristerone

<table>
<thead>
<tr>
<th>Con</th>
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αp204

Con (GFP)

GFP (Con)
Figure 10
p204 protein overcomes the inhibition of the differentiation of P19 murine embryonal carcinoma cells to beating cardiac myocytes by Id proteins
Bo Ding, Chuan-ju Liu, Yan Huang, Jin Yu, Weihua Kong and Peter Lengyel

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