# Max Activity Is Affected by Phosphorylation at Two $NH_2$-Terminal Sites[1]

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## Abstract

**Max is a nuclear phosphoprotein that has a dose-dependent role in regulation of Myc function. The DNA-binding activity of Max homodimers, but not of Myc/Max heterodimers, has been reported to be inhibited by $NH_2$-terminal phosphorylation. (S. J. Berberich and M. D. Cole, Genes & Dev., *6:* 166–176, 1992). Here, we have mapped the $NH_2$-terminal *in vivo* phosphorylation sites of Max to $Ser^2$ and $Ser^{11}$ and show that the $NH_2$ termini of the two major alternatively spliced forms of Max ($p21^{max}$ and $p22^{max}$) are equally phosphorylated despite differences in their amino acid sequences following $Ser^{11}$. A Max mutant deficient in the $NH_2$-terminal phosphorylation was found to inhibit both basal and Myc-induced transcription of a reporter gene more efficiently than the wild-type protein. Similarly, the ability of Myc and Ras to induce transformation was more severely impaired by the mutant. These results indicate that the $NH_2$-terminal phosphorylation diminishes the ability of Max to negatively interfere with Myc function. However, we found no evidence that Max phosphorylation would be regulated during cell growth or differentiation. Similarly, we observed no major cell cycle-dependent changes in the extent of phosphorylation between cell populations fractionated by centrifugal elutriation or by cell cycle inhibitors.**

## Introduction

Max is a small nuclear phosphoprotein (1, 2) that contains a DNA-binding basic region followed by helix-loop-helix and leucine zipper domains which mediate oligomerization. Max can either form homodimers or heterodimerize with proteins of the Myc family (3–5) or the more recently identified proteins Mad and Mxi-1 (6, 7). Since neither Myc nor Mad appears to be able to homodimerize, heterodimerization with Max is presumed to be a requirement for their functional activities (6, 8). Both homo- and heterodimeric protein complexes containing Max can recognize CACGTG or related DNA sequences (1, 6, 9), and thus these different complexes may compete for binding to common DNA targets. Indeed, overexpression of Max has been shown to antagonize Myc-mediated transcriptional *trans*-activation of reporter constructs containing multiple CACGTG sequences (10, 11).

Max can exist in two alternatively spliced forms ($p21^{max}$ and $p22^{max}$), which differ by a 9-amino acid region present only in $p22^{max}$ near its $NH_2$ terminus (1). The use of another alternative exon results in production of COOH-terminally truncated ΔMax polypeptides ($p17^{\Delta max}$ and $p16^{\Delta max}$), which, however, appear to represent a minority of the total cellular Max protein (12, 13). Whereas the full-length Max protein suppresses the ability of Myc to transform REFs[3] in cooperation with Ras, ΔMax, on the contrary, enhances transformation (12). Unlike Myc and Mad, which both have a very short half-life, the Max and ΔMax polypeptides are relatively stable (3, 13). Furthermore, their expression does not appear to change during the cell cycle or during different growth conditions (3, 13, 14).

DNA binding of Max homodimers, but not of Myc/Max or Mad/Max heterodimers, has been shown to be negatively regulated by phosphorylation in the $NH_2$-terminal region of Max[4] (6, 15). This correlates well with the observations that bacterially expressed nonphosphorylated Max polypeptides can efficiently bind to DNA (15, 16), whereas very little binding can be detected by either *in vivo* expressed (15) or *in vitro* translated Max proteins (1, 17) that have been subjected to phosphorylation by cellular kinases. However, the inability of phosphorylated Max to bind DNA can be reversed either by phosphatase treatment or by Myc coexpression (3, 15, 17). CKII has been proposed to be responsible for Max phosphorylation (3, 15), since both the $NH_2$- and COOH-terminal segments of Max contain consensus recognition sites for this enzyme (S/T–X–X–D/E; see Ref. 18). To be able to analyze the role of the phosphorylation in more detail, we identified the *in vivo* phosphorylation sites in the $NH_2$ termini of the Max splice forms. We also tested whether phosphorylation of these sites could affect Max activity and whether the phosphorylation was subjected to growth-dependent regulation.

## Results

**Both Splice Forms of Max and ΔMax Are Phosphorylated *in Vivo*.** To detect phosphoproteins encoded by the *max* gene (Fig. 1*A*), we immunoprecipitated lysates from K562 erythroleukemia cells that had been labeled with $^{32}P_i$ for 4 h. For

Received 11/5/93; revised 12/30/93; accepted 1/4/94.
[1] This work was supported by the Academy of Finland, the Alfred Kordelin Foundation, the Finnish Cancer Organizations, the Finnish Cultural Foundation, the Sigrid Juselius Foundation, and NIH Grant CA20525.
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[3] The abbreviations used are: REF, rat embryo fibroblast; CKII, casein kinase II; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; NP-40, Nonidet P-40; CAT, chloramphenicol acetyltransferase; CnBr, cyanogen bromide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; cDNA, complementary DNA; dm, double mutant.
[4] E. M. Blackwood, unpublished results.

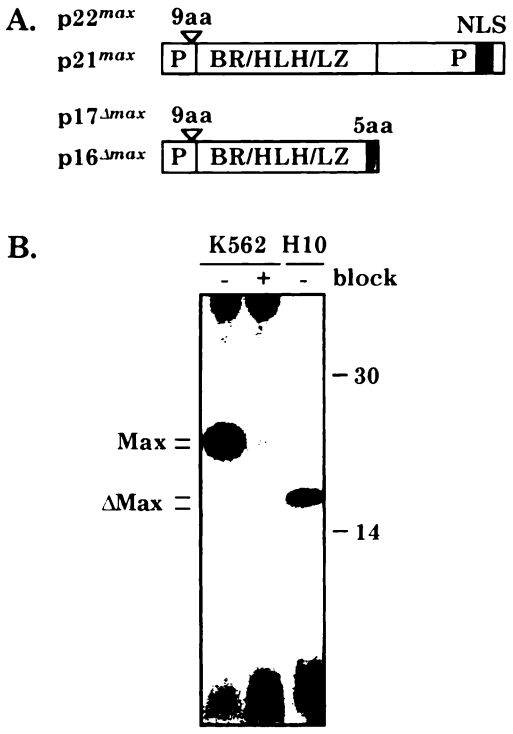



*Fig. 1.* Max and ΔMax proteins are phosphorylated *in vivo*. *A*, schematic comparison of the Max and ΔMax proteins. The positions of the alternatively spliced 9-amino acid regions present in both $p22^{max}$ and $p17^{\Delta max}$, but not in $p21^{max}$ or $p16^{\Delta max}$, are indicated above the proteins. Another alternatively spliced 5-amino acid region specific for ΔMax is *boxed* in its COOH terminus. *BR/HLH/LZ*, basic region/helix-loop-helix/leucine zipper structure; *NLS*, the nuclear localization signal. The approximate locations of clusters of putative phosphorylation sites (*P*) are also indicated. *B*, an SDS-polyacrylamide gel electrophoresis analysis of lysates prepared from $^{32}P_i$-labeled K562 cells and immunoprecipitated with the α-Δ103 Max antiserum in the absence (–) or presence (+) of the corresponding antigen. A similarly treated sample of H10 cells stably overexpressing the $p17^{\Delta max}$ protein is shown as a size marker for the corresponding endogenous protein in K562 cells.

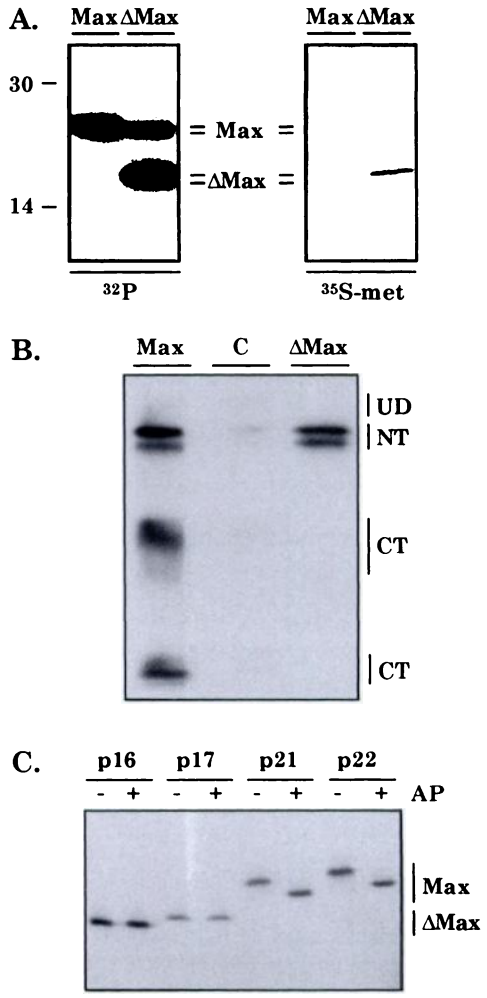



*Fig. 2.* Max is phosphorylated *in vivo* at both its $NH_2$ and COOH termini. *A*, $p22^{max}$ and $p17^{\Delta max}$ immunoprecipitates from transiently transfected COS cells that have been labeled with $^{32}P_i$ (*$^{32}P$*) or with [$^{35}$S]methionine (*$^{35}$S-met*). Note the presence of the endogenous Max of COS cells in all samples. *B*, the $NH_2$-terminal (*NT*) and COOH-terminal (*CT*) tryptic fragments produced from polypeptides excised from the gel shown in *A*. *Lane C*, the endogenous Max polypeptide doublet from the ΔMax-overexpressing cells; *UD*, undigested Max protein. *C*, immunoprecipitates of Max and ΔMax splice forms that have been translated *in vitro* in the presence of [$^{35}$S]methionine. *AP*, samples treated with alkaline phosphatase after immunoprecipitation. Note the mobility shift induced in Max, but not ΔMax polypeptides, by the phosphatase treatment.

immunoprecipitation, we used the α-Δ103 Max antiserum, which has been raised against a β-galactosidase-ΔMax fusion protein, but which recognizes both Max and ΔMax polypeptides (13). Electrophoresis in a 15% SDS-polyacrylamide gel revealed two polypeptide doublets, corresponding to Max (p22 and p21) and ΔMax (p17 and p16) (Fig. 1*B*). Detection of these bands was blocked by preincubation of the antiserum with the corresponding antigen, indicating that the bands are specifically recognized by our antiserum. The upper band of the fainter doublet comigrated with the $p17^{\Delta max}$ protein overexpressed in a stably transfected rat embryo fibroblast-derived cell line. Although this analysis clearly demonstrated that all four polypeptides were expressed and phosphorylated in K562 cells, the amounts of phosphorylated ΔMax polypeptides were strikingly lower than those of Max. This difference could be explained by the lower level of expression of ΔMax and by the absence of the COOH-terminal sequences of Max (Fig. 1*A*), which contain several putative phosphorylation sites.

In order to distinguish between $NH_2$- and COOH-terminal phosphorylation sites in Max, we compared partial tryptic digests of Max and ΔMax. For that purpose, COS cells overexpressing either $p22^{max}$ or $p17^{\Delta max}$ were labeled with $^{32}P_i$ and subjected to immunoprecipitation analysis (Fig. 2*A*). Labeling of parallel plates with [$^{35}$S]methionine revealed that nearly equivalent amounts of $p22^{max}$ and $p17^{\Delta max}$ were synthesized during the 4-h labeling period (Fig. 2*A*). Here, it should be noted that after removal of the initiator methionine (19), there are two remaining methionine residues in Max but only one in ΔMax. The phosphate-labeled protein bands were excised from the gel and subjected to a partial tryptic digestion. As shown in Fig. 2*B*, the $NH_2$-terminal tryptic fragments present in both Max and ΔMax could be separated from the smaller COOH-terminal peptides present only in Max. Furthermore, the mapping revealed that Max is heavily phosphorylated at both its $NH_2$ and COOH termini.

Curiously, when the Max and ΔMax splice forms were translated *in vitro* in reticulocyte lysates in the presence of [$^{35}$S]methionine, we noticed that the electrophoretic mo-

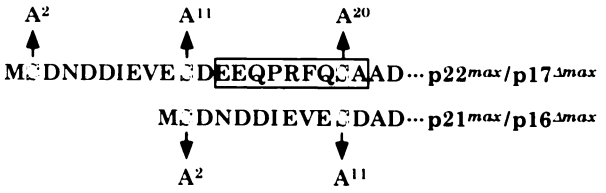



*Fig. 3.* Comparison of the $NH_2$-terminal amino acid sequences preceding the basic regions of the Max and ΔMax splice forms. The *closed box* contains the 9 amino acids specific for $p22^{max}$ and $p17^{\Delta max}$. *Outlined* serine residues, the putative $NH_2$-terminal phosphorylation sites; *arrows*, the serine to alanine mutations that were introduced into the wild-type *max* or *Δmax* expression vectors.

bilities of both $p21^{max}$ and $p22^{max}$ were dramatically decreased during their synthesis (data not shown). These mobility shifts, which could be reversed by treatment with alkaline phosphatase, were apparently due to conformational changes induced by phosphorylation of the COOH-terminal segments of Max proteins, since no such shifts were detected with ΔMax polypeptides containing only $NH_2$-terminal phosphorylation sites (Fig. 2*C*).

**Identification of the $NH_2$-Terminal *in Vivo* Phosphorylation Sites.** According to the deduced amino acid sequence of Max, there are three $NH_2$-terminal serine residues in $p22^{max}$ and two in $p21^{max}$ that could provide sites for the $NH_2$-terminal phosphorylation (Fig. 3). Splicing of the 9-amino acid region changes the amino acid residues COOH-terminal of $Ser^{11}$ and thus can affect recognition of these sites by serine/threonine kinases, such as casein kinase II (see below).

To determine which of the $NH_2$-terminal serine residues are phosphorylated *in vivo*, we separately changed each of them to alanines using polymerase chain reaction mutagenesis (Fig. 3). Since ΔMax lacks the whole COOH-terminal region, with its phosphorylation sites, it provided a useful tool for examination of the $NH_2$-terminal sites. Wild-type or mutant ΔMax splice forms were transiently expressed in COS cells and analyzed for their abilities to incorporate $^{32}P_i$. Whereas none of the mutations alone was able to abolish phosphorylation of $p17^{\Delta max}$ or $p16^{\Delta max}$, no phosphate was incorporated into a $p17^{\Delta max}$ double mutant where both $Ser^2$ and $Ser^{11}$ had been replaced with alanine residues (Fig. 4*A*, and data not shown). These results indicated that both $Ser^2$ and $Ser^{11}$, but not $Ser^{20}$, are phosphorylated. When an equivalent double mutant of $p22^{max}$ was similarly analyzed and compared with the wild-type $p21^{max}$ and $p22^{max}$ proteins, no obvious differences in the overall phosphorylation could be detected. However, partial tryptic mapping revealed that the phosphate in the mutant protein resided exclusively in the COOH-terminal fragments, except for some $NH_2$-terminal background given by the endogenous wild-type protein (Fig. 4*B*). Thus, the extensive COOH-terminal phosphorylation had masked the lack of $NH_2$-terminal phosphorylation in the double mutant. By contrast, $p21^{max}$ and $p22^{max}$ displayed very similar tryptic phosphopeptide patterns, indicating that the presence of the 9-amino acid region does not significantly affect the $NH_2$-terminal phosphorylation *in vivo*. Equivalent amounts of phosphate were also detected in $NH_2$-terminal peptides derived from $p22^{max}$ and $p21^{max}$ immunoprecipitates that had been subjected to *in vitro* phosphorylation by casein kinase II (data not shown), suggesting that CKII or a related kinase is responsible for the $NH_2$-terminal phosphorylation of both Max splice forms.

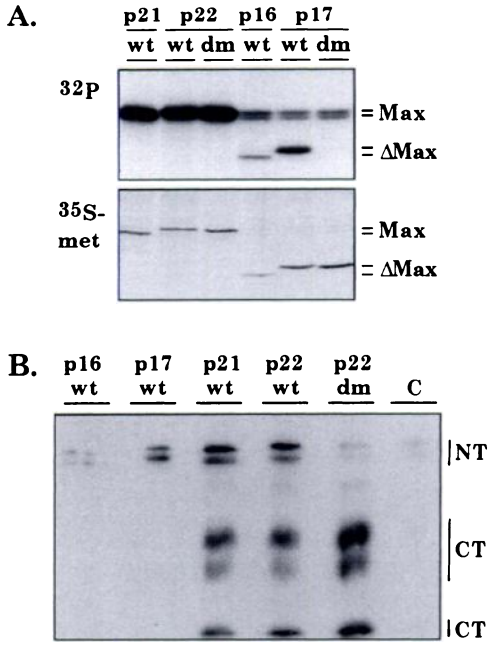
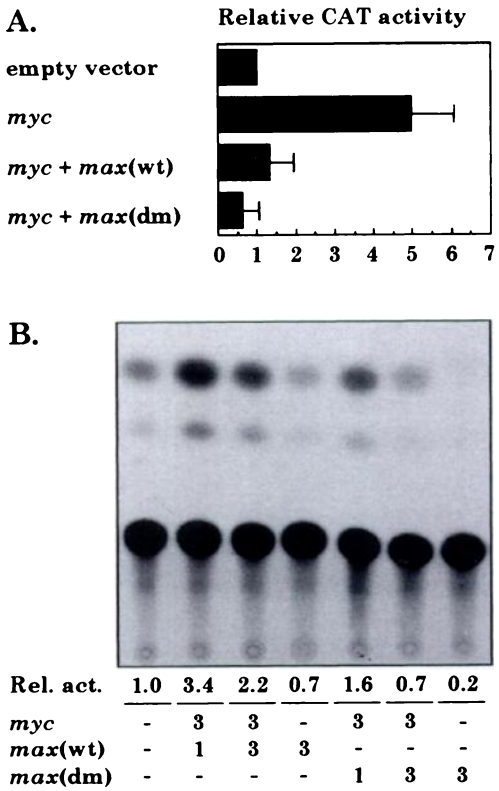



*Fig. 4.* Both $Ser^2$ and $Ser^{11}$ are phosphorylated *in vivo*. *A*, expression and phosphorylation of wild-type (*wt*) Max and ΔMax splice forms and the corresponding double mutants (*dm*), where both $Ser^2$ and $Ser^{11}$ have been replaced with alanine residues. *B*, partial tryptic mapping of indicated polypeptides excised from the gel shown in *A*. Note again the background given by the endogenous Max (*C*) that can also be detected in the $p22^{max}$ (*dm*) sample. *NT*, $NH_2$-terminal; *CT*, COOH-terminal.

**$NH_2$-Terminal Phosphorylation Regulates Max Activity.** Although the ability of Max homodimers to bind *in vitro* to CACGTG-containing oligonucleotides has been shown to be inhibited by $NH_2$-terminal phosphorylation (15), the consequences of this phenomenon *in vivo* have remained unclear. Therefore, we wanted to test whether the $NH_2$-terminal phosphorylation regulates known Max functions such as the abilities of Max to repress Myc-mediated *trans*-activation or transformation. For this purpose, secondary cultures of rat embryo fibroblasts were transfected with *myc* and *max* expression vectors together with the pM4-minCAT reporter construct (10). This construct contains four copies of the CACGTG sequence upstream of a minimal promoter linked to the *CAT* gene. A β-galactosidase gene construct driven by the cytomegalovirus promoter was also included in the transfection mix to control for transfection efficiency. The cells were harvested 48 h after transfection and analyzed for their β-galactosidase and CAT activities. As expected, the Myc-induced 4- to 6-fold *trans*-activation of the reporter construct was severely antagonized by co-overexpression of the $p22^{max}$ protein (Fig. 5*A*). Intriguingly, the $p22^{max}$ double mutant deficient in $NH_2$-terminal phosphorylation was even more repressive than the wild-type protein. A titration experiment (Fig. 5*B*) further demonstrated that, at all doses tested, the Max mutant represses both basal and Myc-induced *trans*-activation 2- to 4-fold more efficiently than wild-type Max.

To determine whether the lack of $NH_2$-terminal phosphorylation could also enhance the repressive effects of Max in a Myc-Ras cotransformation assay, secondary cultures of rat embryo fibroblasts were transfected with *myc* and *max* expression vectors together with the activated c-Ha-*ras* onco-




| Rel. act. | 1.0 | 3.4 | 2.2 | 0.7 | 1.6 | 0.7 | 0.2 |
|---|---|---|---|---|---|---|---|
| *myc* | - | 3 | 3 | - | 3 | 3 | - |
| *max*(wt) | - | 1 | 3 | 3 | - | - | - |
| *max*(dm) | - | - | - | - | 1 | 3 | 3 |

*Fig. 5.* Lack of $NH_2$-terminal phosphorylation increases the ability of $p22^{max}$ to repress Myc-mediated *trans*-activation. *A*, secondary cultures of rat embryo fibroblasts were transiently transfected with the pM4-minCAT reporter construct together with vectors expressing *myc*, wild-type (*wt*) *max*, or a *max* double mutant (*dm*) lacking the $NH_2$-terminal phosphorylation sites. Before assaying for chloramphenicol acetyltransferase activity, the samples were normalized according to their β-galactosidase activities. Results are shown from four separate transfection experiments as a diagram of the induced CAT activities relative to the basal levels observed in cells expressing an empty vector. *Bars*, SD. *B*, results from a titration experiment where REF cultures were transfected with pM4-minCAT and the indicated amounts (μg) of each expression vector. The relative CAT activities (*Rel. act.*) are also shown.

| A. | #1 | #2 | #3 | #4 |
|---|---|---|---|---|
| *myc* 10 + *ras* 10 | 56 | 49 | 20 | 19 |
| + *max* (wt) 2.5 | - | 28 | - | - |
| + *max* (wt) 10 | 33 | 20 | 4 | 5 |
| + *max* (dm) 2.5 | - | 22 | - | - |
| + *max* (dm) 10 | 16 | 10 | 1 | 0 |

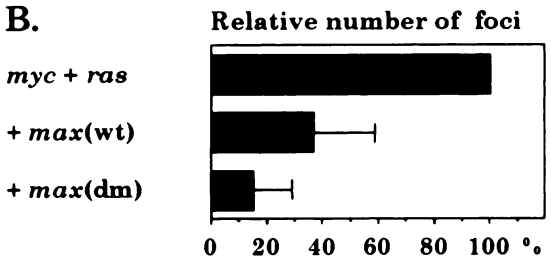



*Fig. 6.* Lack of $NH_2$-terminal phosphorylation enhances the ability of $p22^{max}$ to repress transformation by Myc and Ras. *A*, REF cultures were transfected with the indicated amounts (μg) of each expression vector together with the pSV2-*neo* construct. Transformed foci produced by neomycin-resistant cells were scored 11 to 13 days after transfection. Numbers of foci from four separate transfection experiments are shown. *B*, the numbers of transformed foci shown in *A* from cells transfected with equal amounts of *myc*, *ras*, and *max* or *max* (*dm*) are presented in the diagram relative to those detected in cells transfected with *myc* and *ras* only. *Bars*, SD.

gene. The pSV2-*neo* vector was also included in the transfection mix to allow selection of positive transfectants by neomycin. When the amounts of transformed foci produced by neomycin-resistant cells were scored 11 to 13 days after transfection, it became evident that the ability of Myc and Ras to induce transformation is more severely compromised by co-overexpression of the $p22^{max}$ double mutant that lacks the $NH_2$-terminal phosphorylation sites than by the corresponding wild-type protein (Fig. 6). Since no major differences between the expression levels of the wild-type and mutant proteins could be detected (Fig. 4*A*, and data not shown), our results from both *trans*-activation and transformation assays indicate that phosphorylation reduces the ability of Max to negatively interfere with Myc function.

**$NH_2$-Terminal Phosphorylation of Max Is Not Affected by Changes in Cellular Growth Conditions.** Since phosphorylation appears to influence Max activity, we sought to determine whether phosphorylation of Max is regulated during the cell cycle. We elutriated exponentially growing K562 cells into nine fractions by counterflow centrifugation and labeled equal amounts of cells from each fraction with $^{32}P_i$. The DNA contents of the elutriates were analyzed by fluorescence-activated cell sorting both before and after labeling to determine the initial and final distributions of cells among the $G_1$, S, and $G_2$-M phases (Fig. 7*A*, and data not shown). Immunoprecipitation analysis of phosphorylated Max polypeptides followed by Western blotting (Fig. 7*B*) revealed that the extent of phosphorylation closely correlated with the total amount of Max protein in the different fractions. Thus, the enhanced incorporation of radioactive phosphate could be explained by a general increase in the cellular protein content during the progression of cells from $G_1$ to $G_2$-M. Very similar phosphorylation results were also obtained when K562 cells were exposed overnight to either hydroxyurea or nocodazole to enrich for cell populations at the $G_1$-S boundary or at mitosis, respectively (data not shown).

To be able to compare the extent of $NH_2$-terminal phosphorylation in Max polypeptides immunoprecipitated from the fractionated K562 cells in more detail, the protein bands were excised from the gel and subjected to CnBr cleavage. CnBr was expected to cut $p21^{max}$ and $p22^{max}$ at $Met^{65}$ and $Met^{148}$ or $Met^{74}$ and $Met^{157}$, respectively, and thereby create two large phosphorylated fragments, one containing the $NH_2$-terminal and the other the COOH-terminal phosphorylation sites. The most COOH-terminal fragment consisting of only four amino acids was predicted to remain undetectable owing to its lack of phosphorylation sites. Although CnBr cleaved the Max proteins into the expected fragments, no major cell cycle-dependent differences in either $NH_2$- or COOH-terminal phosphorylation between $p21^{max}$ and $p22^{max}$ were detected (Fig. 7*C*, and data not shown).

We also examined whether phosphorylation of Max is regulated during cell growth or differentiation. For this purpose, secondary cultures of rat embryo fibroblasts were grown for 24 h in the presence of either 0.5% or 10% serum

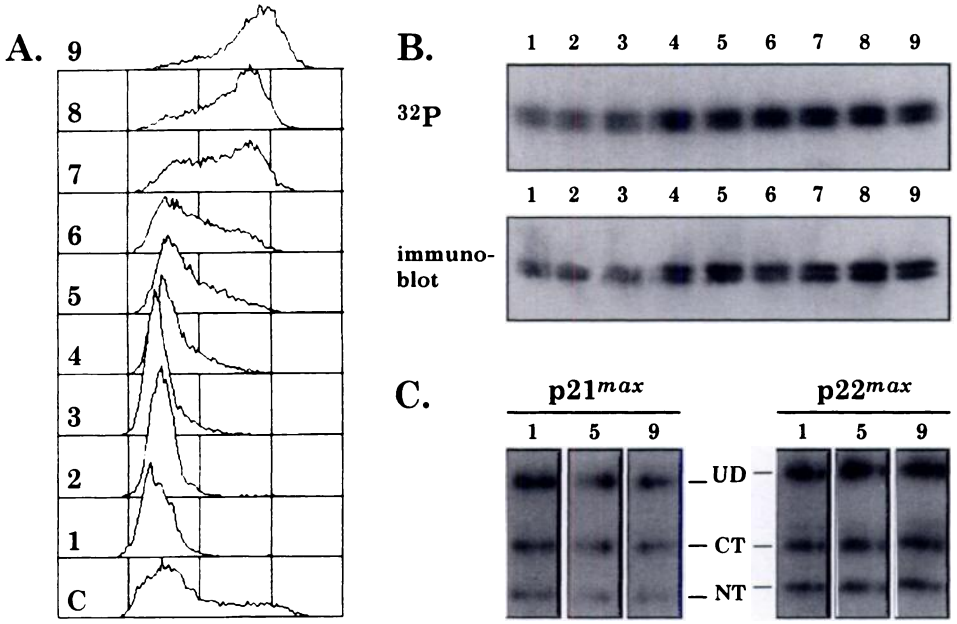



*Fig. 7.* Phosphorylation of Max is not changed during the cell cycle. *A*, exponentially growing K562 cells were elutriated into nine fractions, the DNA contents of which were determined before labeling with $^{32}P_i$. *Abscissa*, increasing DNA content from 2N to 4N. Note that part of the cells moved from one phase to another during the 2-h labeling period. Even then, in the first fraction, more than 85% of the cells still remained in $G_1$, whereas in the last fraction, the amount of cells entering $G_2$-M increased from 70% to 85%. *C*, a sample of asynchronous cells taken before the elutriation was started. *B*, immunoprecipitation analysis of phosphorylated Max polypeptides ($^{32}P$) followed by Western blotting (immunoblot) to determine the steady-state expression levels of the Max splice forms. *C*, partial cyanogen bromide cleavage of samples shown in *B*. *UD*, undigested proteins; *CT*, the COOH-terminally phosphorylated 83-amino acid fragments; *NT*, the $NH_2$-terminally phosphorylated fragments of 64 amino acids in $p21^{max}$ or 73 amino acids in $p22^{max}$.

and were then labeled for 2 h with $^{32}P_i$ in the presence of low or high serum. Immunoprecipitation analysis followed by CnBr digestion revealed no major differences in overall, $NH_2$-terminal, or COOH-terminal phosphorylation of either $p21^{max}$ or $p22^{max}$ among serum-starved, serum-stimulated, or continuously proliferating rat embryo fibroblasts, where the $p21^{max}$ splice form appeared to be overrepresented (Fig. 8*A*, and data not shown). Similarly, no effects on Max phosphorylation were observed when K562 cells were induced toward a megakaryoblastic differentiation pathway by a 2- or 32-h treatment with 3.2 nM TPA (Fig. 8*B*, and data not shown). Taken together, our results indicate that phosphorylation of Max is not subjected to significant cell cycle- or cell growth-dependent regulation.

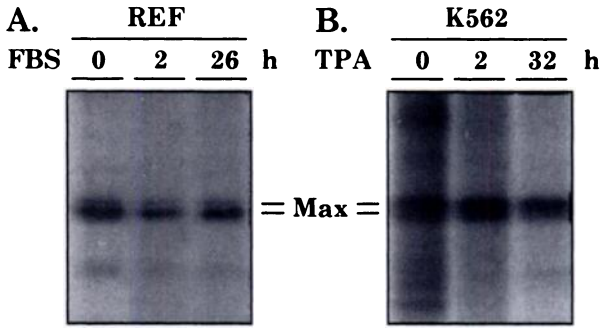



*Fig. 8.* Phosphorylation is not affected by cellular growth conditions. *A*, early passage rat embryo fibroblasts were either serum-starved for 24 h in the presence of 0.5% FBS or allowed to proliferate in the presence of 10% FBS. The cells were then labeled for 2 h with $^{32}P_i$ in the presence of fresh phosphate-free medium supplemented with either 0.5 or 10% FBS. An immunoprecipitation analysis of Max polypeptides phosphorylated in serum-starved (FBS 0 h), serum-stimulated (FBS 2 h), or continuously proliferating (FBS 26 h) cells is shown. *B*, K562 cells were exposed to TPA for the indicated timepoints, labeled for the last 2 h with $^{32}P_i$, and analyzed as in *A*.

## Discussion

Our results indicate that all four polypeptides encoded by the alternatively spliced *max* mRNAs ($p22^{max}$, $p21^{max}$, $p17^{\Delta max}$, and $p16^{\Delta max}$) are phosphorylated *in vivo*. By both partial tryptic mapping and cyanogen bromide cleavage, we were able to distinguish between the $NH_2$-terminal phosphorylated fragments shared by Max and ΔMax and the COOH-terminal ones present only in Max. These analyses revealed that Max is heavily phosphorylated in both its $NH_2$- and COOH-terminal segments. Since $NH_2$-terminal phosphorylation of Max has previously been shown to inhibit its *in vitro* DNA-binding activity (15), our further studies were focused on that region.

Using site-directed mutagenesis, we demonstrate here that $Ser^2$ and $Ser^{11}$ represent the major *in vivo* phosphorylation sites in the $NH_2$ terminus of Max. A similar conclusion has also been obtained in a concurrent study using two-dimensional analyses of Max phosphopeptides (19). Our results indicate that although the presence or absence of the 9-amino acid alternatively spliced region changes the amino acid composition COOH-terminal of $Ser^{11}$, this does not result in major differences in *in vivo* phosphorylation between the two Max splice forms. Furthermore, since casein kinase II is able to phosphorylate the $NH_2$-terminal sites *in vitro* in both $p21^{max}$ and $p22^{max}$, this suggests that CKII or a related kinase is most likely to be responsible for the *in vivo*

phosphorylation of these sites. This is supported by the lack of obvious consensus sites for other known serine/threonine kinases such as glycogen synthase kinase 3 or mitogen-activated protein kinases in the $NH_2$ terminus of Max and is consistent with our inability to phosphorylate Max *in vitro* by glycogen synthase kinase 3.[5]

Our studies on the functional consequences of the $NH_2$-terminal phosphorylation revealed that a mutant $p22^{max}$ protein that lacks the two $NH_2$-terminal phosphorylation sites represses both basal and Myc-induced *trans*-activation of a reporter construct more efficiently than the corresponding wild-type protein. Similarly, the inhibitory effects of Max overexpression in the Myc-Ras cotransformation assay are enhanced by the absence of $NH_2$-terminal phosphorylation. This latter result is consistent with a conclusion from a previous analysis where $Ser^2$ and $Ser^{11}$ of $p21^{max}$ were replaced with alanine and glycine residues, respectively (17), although, in that study, the effects of the wild-type and mutant $p21^{max}$ proteins were not directly compared. Taken together, these results indicate that phosphorylation diminishes the ability of both Max splice forms to negatively interfere with Myc function. Since phosphorylation has been shown to inhibit DNA binding of Max homodimers, but not of Myc/Max or Mad/Max heterodimers (6, 15, 17),[4] our results may be explained by the reduced ability of phosphorylated Max homodimers to compete with Myc/Max heterodimers for binding to the CACGTG sequences. This, in turn, could be due to phosphorylation-induced conformational changes diminishing the DNA binding affinity or stability of Max homodimers. These conclusions are supported by the observations that, in the absence of phosphorylation, there are no significant differences in the intrinsic DNA binding affinities between wild-type Max and phosphorylation-deficient mutants similar to those used in our study (19).[4] However, it remains formally possible that the $NH_2$-terminal serine to alanine mutations modulate other properties of Max besides DNA binding and thereby increase its inhibitory effects on Myc function.

In contrast to previous data, it was recently demonstrated that the $NH_2$-terminal phosphorylation of Max does not affect the steady-state level of DNA binding but rather results in a concomitant increase in both on- and off-rates of Max polypeptides from their DNA targets (19). Furthermore, phosphorylation by CKII was shown to affect Max homodimers and Myc/Max heterodimers to a similar extent. However, if phosphorylation only increases the exchange rates and thus does not influence the average occupancies of binding sites by homo- or heterodimeric protein complexes, one would not expect to see any differences in Max activities whether or not its $NH_2$-terminal sites are phosphorylated. Nonetheless, the results from our *in vivo* assays indicate that lack of $NH_2$-terminal phosphorylation enhances the repressive effects of Max on Myc function, which we presume to be due to the action of Max homodimers. In contrast, we found no evidence for corresponding phosphorylation-dependent changes in the activity of Myc/Max heterodimers. Although in partial disagreement with the data of Bousset and coworkers (19), our conclusions are in agreement with results from several other laboratories (see above). Although it is difficult for us to determine the reasons for the observed differences in the effects of CKII phosphorylation on the *in vitro* DNA-binding activities of Max homodimers *versus* Myc/Max or Mad/Max heterodimers, differences in experimental conditions provide the most likely explanation, especially since the gel shift assay seems to be very sensitive to even slight changes.[6] Also, it is possible that the four additional amino acids in the $NH_2$ termini of the Max proteins produced from the constructs of Bousset and coworkers (19) interfere with the effects of CKII.

Since the relative amounts of Max homodimers and Myc/Max heterodimers vary depending on the proliferation state of cells (3, 14), it is tempting to speculate that phosphorylation of Max and hence its DNA-binding activity could be regulated in a cell growth- or cell cycle-dependent manner. However, our results provide evidence against major changes in Max phosphorylation. Our analysis with rat embryo fibroblasts revealed that the $NH_2$- and COOH-terminal segments of both $p21^{max}$ and $p22^{max}$ are phosphorylated to equivalent extents irrespective of whether the cells are continuously proliferating, arrested in $G_0$, or restimulated to enter into $G_1$. Very similarly, no effects on phosphorylation can be detected when K562 cells are induced to differentiate. Furthermore, our results both from cell cycle-fractionated K562 cells and from cells treated with cell cycle inhibitors indicate that the relative amounts of phosphate in the $NH_2$- and COOH-terminal segments of the Max polypeptides remain unchanged during the distinct phases of the cell cycle.

These findings are similar to results from a recent study on the serum response factor. The DNA-binding activity of the serum response factor has also been shown to be affected by phosphorylation, but the phosphorylation state of the protein does not change upon growth factor treatment (20). Most probably, both the $NH_2$- and COOH-terminal segments of Max become fully phosphorylated during their *in vivo* synthesis. This is supported by the functional differences observed between the wild-type Max and the corresponding mutant deficient in $NH_2$-terminal phosphorylation, and by the electrophoretic mobility shifts induced by COOH-terminal phosphorylation that were detected during *in vitro* translation of Max polypeptides. However, our assays were not sensitive enough to detect differences in occupancies of single phosphorylation sites. Also, our data do not rule out the possibility that a minor population of the Max polypeptides remains unphosphorylated or becomes dephosphorylated by cellular phosphatases.

In conclusion, our results from both *trans*-activation and transformation assays indicate that the *in vivo* phosphorylation of $Ser^2$ and $Ser^{11}$ residues in the $NH_2$ terminus of Max reduces its ability to negatively interfere with Myc function. However, the lack of regulation of Max phosphorylation suggests that the functional activities of Max are mainly determined by the homo- or heterodimeric complexes that it forms under various growth conditions.

## Materials and Methods

**Cell Culture and Transfections.** K562 and COS-7 cells were grown in RPMI 1640 and DMEM, respectively, supplemented with 5% FBS. Induction of megakaryoblastic differentiation of K562 leukemia cells was started as previously described (21) by exposing exponentially growing cells to 3.2 nM TPA. Within 24 h, the TPA-treated cells were expected to cease to proliferate and start to express

[5] P. J. Koskinen, unpublished results.

[6] E. M. Blackwood, personal communication.

differentiation-specific proteins (22). Transient transfections of COS cells were carried out by the DEAE-dextran method (23). REFs were prepared from 13- to 15-day-old Sprague-Dawley rat embryos, grown in DMEM supplemented with 10% FBS, and passaged once before transfecting them by the calcium phosphate precipitation technique (24).

**Expression Vectors.** Serine to alanine mutations were introduced into pSV-*max* or pSV-Δ*max* expression vectors (12) by using mutated oligonucleotides in polymerase chain reactions. In pSV-*max* (dm) and pSV-Δ*max* (dm) constructs, both $Ser^2$ and $Ser^{11}$ have been replaced with alanines. All mutations were verified by sequencing of the coding regions. pLTR-Tc-*myc* contains a full-length c-*myc* cDNA that has been transferred as a *Bam*HI-*Eco*RI fragment from pT7-Tc-*myc* plasmid (12) into pLTRpoly vector (25).

**Production of the α-Δ103 Max Antiserum.** The protein coding region of the Δ*max* cDNA was cloned into the pEX-1 vector (26) in frame with sequences encoding β-galactosidase. The fusion protein was expressed in *Escherichia coli*, purified in a soluble form from a preparative agarose gel (Prosieve; FMC BioProducts), and used for immunization of rabbits. The resulting polyclonal antiserum immunoprecipitates both ΔMax and Max proteins (13).

***In Vitro* Transcription and Translation.** pGEM constructs containing *max* or Δ*max* cDNAs (12) were transcribed *in vitro* using SP6 polymerase in the presence of GpppG and then translated in rabbit reticulocyte lysates (Promega) in the presence of [$^{35}$S]methionine.

**Immunoprecipitation Analyses.** To detect Max or ΔMax proteins, cells were labeled for 2 to 4 h with either [$^{35}$S]methionine (150 μCi/ml) or $^{32}P_i$ (300 μCi/ml) and lysed in AB buffer [20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% NP-40, 0.5% SDS, and 0.5% DOC] containing proteinase (0.3% aprotinin) and phosphatase inhibitors (4 mM NaF and 50 μM sodium orthovanadate). Sonicated cell lysates were cleared by centrifugation and immunoprecipitated with the α-Δ103 Max antiserum in the presence of Protein A-Sepharose particles. Immunoprecipitates were washed twice with AB buffer, once with high-salt buffer [10 mM Tris-HCl (pH 7.4), 2 M NaCl, 1% NP-40, and 0.5% DOC], and twice with radioimmunoprecipitation assay buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1% DOC] and were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

For dephosphorylation, Max or ΔMax immunoprecipitates were washed twice with AB buffer, once with high-salt buffer, once with TBS [50 mM Tris-HCl (pH 7.4), and 150 mM NaCl] with 1% NP-40, and once with TBS without NP-40. The samples were then incubated with calf intestinal alkaline phosphatase (Promega) in TBS for 20 min followed by two washes with radioimmunoprecipitation assay buffer.

**Proteolytic Digestions.** For a partial tryptic digestion, the protein bands of interest were excised from a dried but unfixed gel, allowed to swell in 0.1× electrophoresis sample buffer [25 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, and 2.5% 2-mercaptoethanol], and placed horizontally into wells of a 19% SDS-polyacrylamide gel containing 1 μg of a crude trypsin preparation (Difco; 1:250) in 1× sample buffer.

For cyanogen bromide cleavage, the gel slices were allowed to swell in water and incubated with a 1:10 dilution of 5 M CnBr solution (Aldrich) in a buffer containing 10% HCOOH, 0.1 M HCl, and 0.2% 2-mercaptoethanol for 1 h in the dark at room temperature. After cleavage, the gel slices were washed twice in water and once in 0.25 M Tris-HCl (pH 6.8), incubated for 10 min in 1× sample buffer, and then placed horizontally into wells of a 15% SDS-polyacrylamide gel.

***Trans*-activation Assays.** Secondary cultures of rat embryo fibroblasts were transfected with 10 μg of DNA including 1 to 3 μg of the pLTR-Tc-*myc*, pSV-*max*, or pSV-*max* (dm) expression vectors or the empty pSV-poly vector, 3 μg of the pM4-minCAT reporter (10), and 1 μg of the cytomegalovirus-β-galactosidase vector (pCH110; Pharmacia LKB). β-Galactosidase activities were measured as described by Geballe and Mocarski (27). Chloramphenicol acetyltransferase activity assays were carried out using the thin-layer chromatographic method of Gorman and coworkers (28).

**REF Cotransformation Assays.** Secondary cultures of rat embryo fibroblasts were transfected with 31 μg of DNA including 1 μg of pSV2*neo* (American Type Culture Collection no. 37149) and 2.5 to 10 μg of each pLTR-Tc-*myc*, pGEJ(6.6) (12) and either pSV-*max*, pSV-*max* (dm), or pSV-poly. The transfected cells were split once in a 1:5 ratio and grown in DMEM supplemented with 5% FBS and 0.2 mg/ml G418 (GIBCO-BRL). Medium was replenished every third day, and transformed foci were scored 11 to 13 days after transfection.

**Cell Cycle Analyses.** Exponentially growing K562 cells were elutriated by counterflow centrifugation into 10 fractions. Then $10^7$ cell samples of each fraction were labeled with $^{32}P_i$ for 2 h and subjected to immunoprecipitation analysis with the rabbit polyclonal anti-glutathione *S*-transferase-MaxC124 antiserum (1). The steady-state levels of Max protein were determined by Western blotting using the enhanced chemiluminescence detection system of Amersham. The DNA contents of the elutriated fractions were analyzed both before and after labeling by fluorescence-activated cell sorting analysis of propidium iodide-stained cells.

## Acknowledgments

We thank David Litchfield and Erwin Krebs for purified casein kinase II; Sandra Jo Thomas, Birgitta Tjäder, and Ulla Pirvola for preparation of rat embryos; Andrew Koff for advice on elutriation; Elizabeth Blackwood and Leo Kretzner for reagents, discussions, and critical comments on the manuscript; and Bernard Lüscher for communication of unpublished data.

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