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The N-Myc oncoprotein is associated *in vivo* with the phosphoprotein Max(p20/22) in human neuroblastoma cells

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Proteins encoded by the proto-oncogenes c-*myc*, L-*myc*, and N-*myc* contain at their carboxy-terminus a tripartite segment comprising a basic DNA binding region (BR), a helix-loop-helix (HLH) and a leucine zipper motif (Zip), that are believed to be involved in DNA binding and protein-protein interaction. The N-Myc oncoprotein is overexpressed in certain human tumors that share neuroectodermal features due to amplification of the N-*myc* gene. Using a monoclonal antibody directed against an N-terminal epitope of the N-Myc protein in immunoprecipitations performed with extracts of neuroblastoma cells, two nuclear phosphoproteins, p20/22, forming a hetero-oligomeric complex with N-Myc are identified. Both proteins are phosphorylated by casein kinase II *in vitro*. By partial proteolytic maps we show that p20 and p22 are structurally related to each other and that p20 is identical with Max, a recently described *in vitro* binding partner of myc proteins. Time course experiments show the presence of the complex in cellular extracts immunoprecipitated within a 5 min interval after the preparation of the cell extract. While the expression of N-*myc* is restricted, expression of both Max(p20/22) and the murine homolog Myn(p20/22) was observed in cells of diverse human and murine embryonal lineages as detected by heterologous complex formation. By introduction of expression vectors containing the wild type N-*myc* gene or N-*myc* genes with in frame deletions or point mutations into recipient cells and subsequent immunoprecipitation of the resulting N-Myc proteins we show that the HLH-Zip region is essential to the formation of the N-Myc-p20/22 complex.

Key words: amplification/casein kinase II/N-*myc*/nuclear oncogenes/protein dimerization

Introduction

Members of the *myc* gene family, of which the best characterized are c-*myc*, N-*myc* and L-*myc*, are often altered in animal and human cancer cells and it is thought that these alterations contribute to tumorigenesis (for recent reviews see Bishop 1991; Lüscher and Eisenman, 1990). The N-*myc* gene was originally identified due to its frequent amplification in advanced human neuroblastomas (Kohl *et al.*, 1983; Schwab *et al.*, 1983). Subsequent studies revealed amplification of N-*myc* also in small cell lung cancers (Nau *et al.*, 1986), retinoblastomas (Lee *et al.*, 1984), malignant gliomas (Bigner *et al.*, 1988) and astrocytomas (Garson *et al.*, 1985), all tumors that share neuroectodermal features (Schwab, 1990). In addition to amplification in human cancers, N-*myc* has been found activated through insertion of a Moloney murine leukemia provirus in lymphomas of the rat (van Lohuizen *et al.*, 1989) and by hepadnavirus insertion in liver tumors of the woodchuck (Fourel *et al.*, 1990).

The N-*myc* gene encodes two polypeptides (p58/60$^{N\text{-}Myc}$; Mäkelä *et al.*, 1989) that result from alternative usage of two translation initiation sites. In SDS-polyacrylamide gels four closely migrating polypeptide bands (p58-64$^{N\text{-}Myc}$) are observed, a heterogeneity which results from post-translational modification by phosphorylation (Mäkelä *et al.*, 1989). The N-Myc protein is located in the nucleus of the cell, phosphorylated by casein kinase II (Hamann *et al.*, 1991), has an affinity for DNA *in vitro* and a short half-life (Ramsay *et al.*, 1986). Inspection of the protein sequence deduced from cDNA clones revealed that the N-Myc protein, like other myc proteins, shares structural motifs with known transcription factors some of which like Jun or Fos are also products of proto-oncogenes (Kouzarides and Ziff, 1988). These motifs comprise a basic DNA binding region (BR), an α-helical protein-protein interaction domain known as helix-loop-helix (HLH) and a leucine zipper motif (Zip) (Kouzarides and Ziff, 1988; Landschulz *et al.*, 1988; Murre *et al.*, 1989a). Dimerization either via the HLH- or Zip-domain is a critical determinant of function and permits the adjacent basic region to form a DNA binding surface (Vinson *et al.*, 1989; Jones 1990). The specificity of dimer formation and DNA binding is determined through the type and combination of motifs present within the proteins and is therefore limited to certain classes of proteins (Jones, 1990; Johnson and McKnight, 1989). Protein-protein interaction via the HLH motif is mediated by means of hydrophobic and charged amino acids (Murre *et al.*, 1989a) whereas in the Zip motif the leucines align along one face of the α-helix allowing the leucine residues to interdigitate by hydrophobic interactions to form dimers in a coiled-coil fashion (O'Shea *et al.*, 1989). The stability and specificity of dimers formed by leucine zippers is also determined through the many charged residues within the Zip motifs (Kouzarides and Ziff, 1988).

Proteins containing these motifs may form homo- or heterodimers and are sometimes members of polyprotein complexes which regulate gene expression by binding to DNA sequences embedded in promoters, enhancers and silencers (Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Murre *et al.*, 1989b; Blackwell and Weintraub, 1990). In the myc proteins all three motifs are arranged in tandem at the C-terminus forming a tripartite segment termed the BR-HLH-Zip motif. In this respect the myc proteins are distinguishable from other classes of nuclear proteins that contain either a BR-Zip motif like Jun and Fos, which are

the active components of the transcriptional factor AP-1 (Bohmann *et al.*, 1987; Angel *et al.*, 1988) or a BR-HLH motif like MyoD1, a known muscle determination factor (Tapscott *et al.*, 1988).

In an elegant approach using an *in vitro* assay, Blackwood and Eisenman recently reported the isolation of a cDNA coding for a BR-HLH-Zip protein with a molecular mass of ~21 kDa, dubbed Max, which can associate with the Myc protein *in vitro* through the HLH-Zip domain (Blackwood and Eisenman, 1991). These authors also showed that Max forms heterodimeric complexes with the L-Myc and N-Myc protein *in vitro* and that the Myc–Max complex binds to DNA in a sequence-specific manner. In another report it was shown that Myn, the murine homolog of Max, binds to the c-Myc protein through the HLH-Zip motif *in vitro* (Prendergast *et al.*, 1991). Association of Myn with c-Myc stimulates methylation-sensitive DNA binding as well as *ras* co-transformation (Prendergast *et al.*, 1991).

For the N-Myc protein there is little known about the possible functions of the BR-HLH-Zip region. Enhanced expression of N-*myc* cooperates with mutationally altered $p21^{RAS}$ in *in vitro* transformation (Schwab *et al.*, 1985), and it was shown that deletions or single amino acid substitutions in the C-terminal region led to a drastic reduction in oncogenic potential (Ueno *et al.*, 1988; Nakajima *et al.*, 1989). These results clearly point to the importance of an intact BR-HLH-Zip region for protein function.

Because N-Myc contains amino acid sequence motifs known to be involved in protein–protein interactions, we have searched for cellular proteins that are associated with the N-Myc protein *in vivo*. We first generated high affinity monoclonal antibodies (mAb) directed against N-terminal epitopes of $p58\text{-}64^{N\text{-}Myc}$, and then analyzed by immunoprecipitation extracts of human neuroblastoma cells that express high levels of N-Myc protein consequent to amplification for *in vivo* associated proteins. Here we report that the N-Myc oncoprotein associates with two nuclear phosphoproteins, p20/22, in cellular extracts of human neuroblastoma cells that are identical with the Max protein, an *in vitro* binding partner for myc proteins. Moreover the experimental conditions we used allow us to conclude that Max(p20/22) also associates *in vivo* with the N-Myc oncoprotein suggesting that the complex is of biological

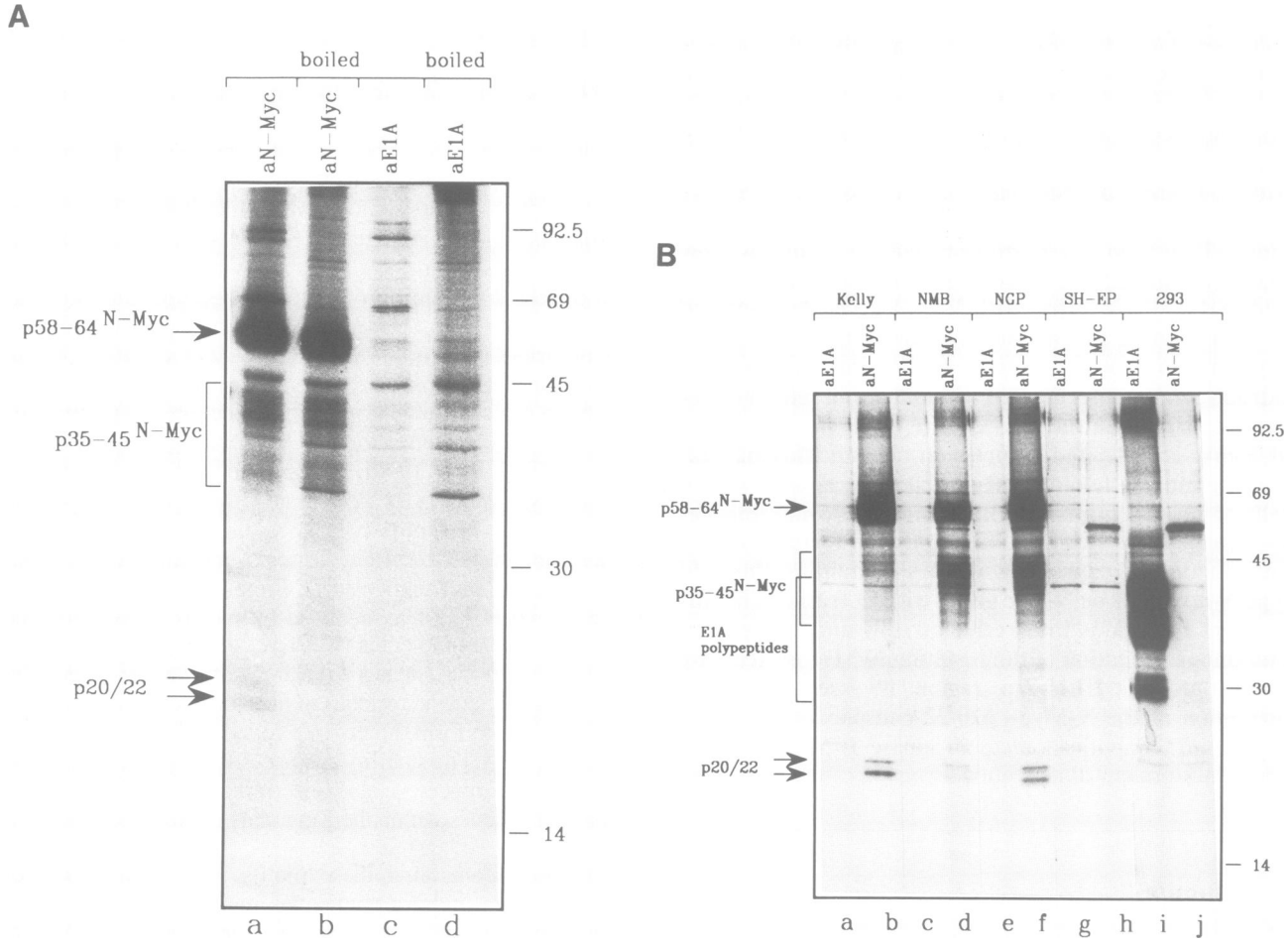

Fig. 1. Identification of the N-Myc–p20/22 complex in human neuroblastoma cells. **A**, Extracts from [^{35}S]methionine-labelled cultures of human Kelly neuroblastoma cells (lanes a, b) were immunoprecipitated with monoclonal antibody aN-Myc (anti-N-Myc; IgG_{2a}) specific for an N-terminal epitope of the N-Myc protein, or with aE1A (anti-adenovirus E1A polypeptides) as a control monoclonal antibody (lanes c, d). Immunoprecipitates from boiled extracts (lanes b, d) using the same monoclonal antibodies served as controls. Proteins were separated on a 12.5% SDS–polyacrylamide gel. Size markers are in kDa. **B**, Extracts from [^{35}S]methionine-labelled cultures of human neuroblastoma cell lines Kelly (lanes a, b), NMB (lanes c, d), NGP (lanes e, f) and as a control the human neuroblastoma cell line SH-EP (lanes g, h) and the human cell line 293 transformed with DNA from human adenovirus type 5 (Graham *et al.*, 1977) (lanes i, j) were used in the immunoprecipitation with either aN-Myc monoclonal antibody or aE1A as control. Proteins were separated on parallel 12.5% SDS–polyacrylamide gels. Size markers are in kDa.

significance. Further, by analyzing extracts of cells that express mutant N-Myc proteins after transfection we mapped the site of association between N-Myc and p20/22 to the HLH-Zip region.

Results

p58-64$^{N\text{-}Myc}$ is associated with p20/22

To identify proteins associated with N-Myc we generated monoclonal antibodies against the N-Myc protein. After immunization with a bacterially expressed β-galactosidase−N-Myc fusion protein, a series of monoclonal antibodies specific for N-terminal epitopes of N-Myc was obtained (unpublished data). In preliminary immunoprecipitations using the anti-N-Myc monoclonal antibody (aN-Myc) we employed cells of the human neuroblastoma line Kelly which show high expression of p58-64$^{N\text{-}Myc}$ as the result of ~150-fold amplification of the N-*myc* gene. Cells were metabolically labelled with L-[^{35}S]methionine lysed under mild conditions and nuclei extracted with buffers containing low concentrations of salt and the detergent NP-40, and the N-Myc protein was immunoprecipitated. After washing, the immunoprecipitates were fractionated by SDS−PAGE. Under these conditions large amounts of the p58-64$^{N\text{-}Myc}$ are readily detected (Figure 1A, lane a) as well as several additional bands. Some of the latter, as shown by Western blot analysis, represent N-Myc degradation products which have an apparent molecular mass of 35−45 kDa (data not shown). Of particular interest were two proteins migrating as a doublet at around 20 and 22 kDa. The doublet was completely absent when the extracts were denatured by boiling in SDS prior to incubation with antibody, although p58-64$^{N\text{-}Myc}$ as well as its degradation products were still immunoprecipitated (lane b). This indicates that the epitope recognized by aN-Myc is resistant to heat denaturation and at the same time suggests that the presence of p20/22 in the immunoprecipitates is not the result of a cross-reaction. As further evidence of this, Western blot analysis of ^{35}S-labelled N-Myc complexes, which were purified by immunoprecipitation and probed with aN-Myc revealed both p58-64$^{N\text{-}Myc}$ and the degradation products, but showed no signal for p20/22. The presence of p20/22 could nevertheless be shown by autoradiography of the same blot (data not shown). Using aE1A (anti-adenovirus E1A polypeptides) as control monoclonal antibody, which is of the same isotype (IgG_{2a}) as aN-Myc no co-precipitating proteins in the 20/22 kDa range were detected (lanes c, d). Two other neuroblastoma cell lines, NMB, and NGP gave similar results (Figure 1B, lanes d, f), as did cell lines derived from other tumors which carry amplified N-*myc*, such as retinoblastoma and small cell lung cancer (data not shown). In contrast in the tumor cell line SH-EP and 293 cells without detectable amounts of p58-64$^{N\text{-}Myc}$, there was no precipitation of p20/22 (lanes h, j).

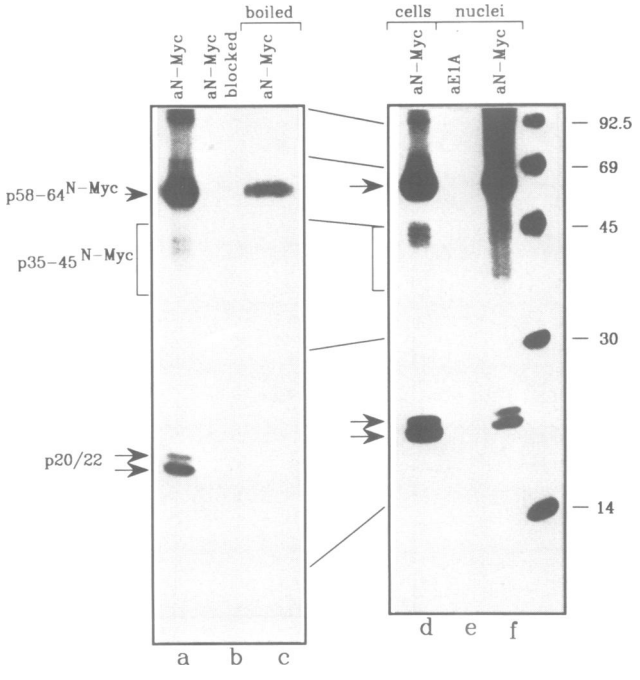

Fig. 2. p20 and p22 are nuclear phosphoproteins associated with N-Myc. Extracts from [^{32}P]orthophosphate-labelled cultures of human Kelly neuroblastoma cells were immunoprecipitated either with aN-Myc (lane a) or aN-Myc blocked with bacterially expressed β-galactosidase−N-Myc fusion protein aN-Myc blocked (lane b) as a control. As a further control, boiled extracts of Kelly neuroblastoma cells were immunoprecipitated with aN-Myc (lane c). To demonstrate the nuclear localization of p20/22 and to compare the relative amounts of immunoprecipitable p58-64$^{N\text{-}Myc}$−p20/22 complex in extracts of total cells and isolated nuclei, either extracts of 1×10^6 Kelly cells (lane d) or 1×10^6 nuclei (lane f) were immunoprecipitated with aN-Myc monoclonal antibody. As a control aE1A monoclonal antibody was used in extracts of nuclei (lane e). Proteins were separated on 12.5% SDS−polyacrylamide gels. Size markers are in kDa.

Both p20 and p22 showed very low incorporation of [^{35}S]methionine as compared with N-Myc. This could be due to fewer methionine residues, to non-stochiometric association with p58-64$^{N\text{-}Myc}$ or to a low rate of translation. The relatively weak signals after [^{35}S]methionine labelling made the more detailed analysis of p20/22 impractical due to the very long autoradiographic exposure times required. We therefore investigated if p20/22 could be metabolically labelled with [^{32}P]orthophosphate using Kelly cells in our preliminary studies. Using the aN-Myc monoclonal antibody we could readily detect the p20/22 doublet on autoradiographs (Figure 2, lane a) and show that the p20/22 doublet is modified by phosphorylation.

After ^{32}P-labelling (e.g. Figure 2, lane a) it is apparent that the signal from p22 is weaker than the one from p20. This may indicate that there is less p22 bound to N-Myc due to lower affinity or alternatively that p22 is phosphorylated to a lesser extent. It seems more likely that both proteins have different affinities for N-Myc since the difference in the autoradiographic signal intensity can also be observed in immunoprecipitations performed after ^{35}S-labelling (compare Figure 1B, lane b with Figure 2, lane a). Another explanation for the observed heterogeneity of the p20/22 doublet may be that these two proteins are present at different levels. p20/22 could not be detected in immunoprecipitations when aN-Myc was blocked with bacterially expressed fusion protein (Figure 2, lane b) or when the extracts were heated at 100°C (boiled) in the presence of SDS (lane c). When nuclei were separately isolated before the preparation of extracts, p20/22 could be immunoprecipitated (lane f). These data suggest that p20/22 is localized in the nucleus, although at this point we cannot exclude that some fraction of the protein also resides in the cytoplasm.

To address the question as to whether the N-Myc−p20/22

complex is formed *in vitro* during the immunoprecipitation or if it pre-exists *in vivo* in the cell, we immunoprecipitated p58-64$^{N\text{-}Myc}$ at various time intervals after the extract preparation. p20/22 can be detected readily in extracts immunoprecipitated only 5 min after preparation (Figure 3). This observation indicates that the N-Myc−p20/22 complex exists *in vivo* in the cell.

The N-Myc−p20/22 complex is phophorylated by casein kinase II in vitro

The N-Myc protein is phosphorylated by casein kinase II (CK-II) *in vivo* and *in vitro* (Hamann *et al.*, 1991). To study whether p20/22 is also an *in vitro* substrate of CK-II, extracts from unlabelled Kelly cells were prepared and phosphorylated *in vitro*. As shown in Figure 4, p20/22 is phophorylated in association with p58-64$^{N\text{-}Myc}$ by CK-II *in vitro*. Comparison of *in vivo* phosphorylated p20/22, which was isolated from ^{32}P-labelled Kelly cells, with *in vitro* phosphorylated p20/22 revealed an identical pattern of phosphorylation. Under both labelling conditions the signal of p22 was weaker than that of p20. This observation is in accordance with ^{35}S-labelled p20/22 (see Figure 1B, lane b) where the signals of the respective proteins show different intensities. The significant difference in signal strength between p20 and p22 obtained after phosphorylation *in vivo* as well as *in vitro* indicates once more that p20 and p22 either are differentially phosphorylated, have different binding affinities to the N-Myc protein or are expressed in different levels.

Among the proteins adsorbed to protein A−Sepharose beads during immunoprecipitation, only p58-64$^{N\text{-}Myc}$ and p20/22 are phosphorylated by CK II *in vitro*. The specificity of the reaction is also demonstrated by the lack of phosphorylation of the IgG heavy and light chain as well as by the fact that immunoprecipitated proteins encoded by the retinoblastoma gene *Rb1* (Lee *et al.*, 1987) which was tested for comparison, were not phosphorylated *in vitro* by CK-II (Figure 4).

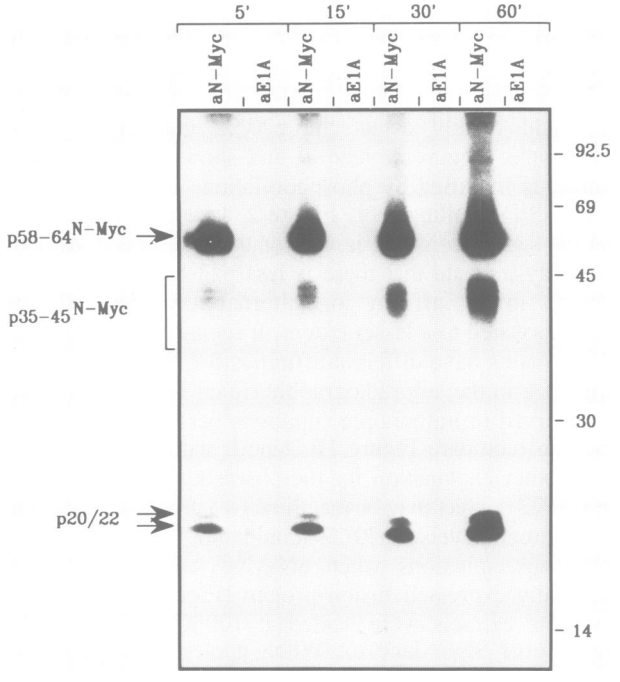

Fig. 3. Immunoprecipitations of the N-Myc−p20/22 complex for various time periods. Extracts from [^{32}P]orthophosphate-labelled cultures of Kelly neuroblastoma cells were precipitated for 5, 15, 30 or 60 min as indicated with either aN-Myc or aE1A monoclonal antibody as a control. Proteins were separated on a 12.5% SDS−polyacrylamide gel. Size markers are in kDa.

N-Myc associated p20 is identical to Myc associated Max and p22 is a related protein

In order to determine the relationship between p20/22 and the Max protein, we compared partial proteolytic maps of *in vitro* phosphorylated p20/22 and p21Max. The p21Max protein obtained by *in vitro* translation from pVZ1p21Max (Blackwood and Eisenman, 1991) was mixed with *in vitro* translated N-Myc protein. The N-Myc−p21Max complex, which can be reconstituted under *in vitro* conditions (Blackwood and Eisenmann, 1991; and data not shown) was immunoprecipitated using the aN-Myc monoclonal antibody and *in vitro* phosphorylated by CK-II. Analysis of the amino acid sequence of Max revealed at least six putative CK-II phosphorylation sites. p20/22 was isolated by immunoprecipitation of extracts from non-labelled Kelly cells and

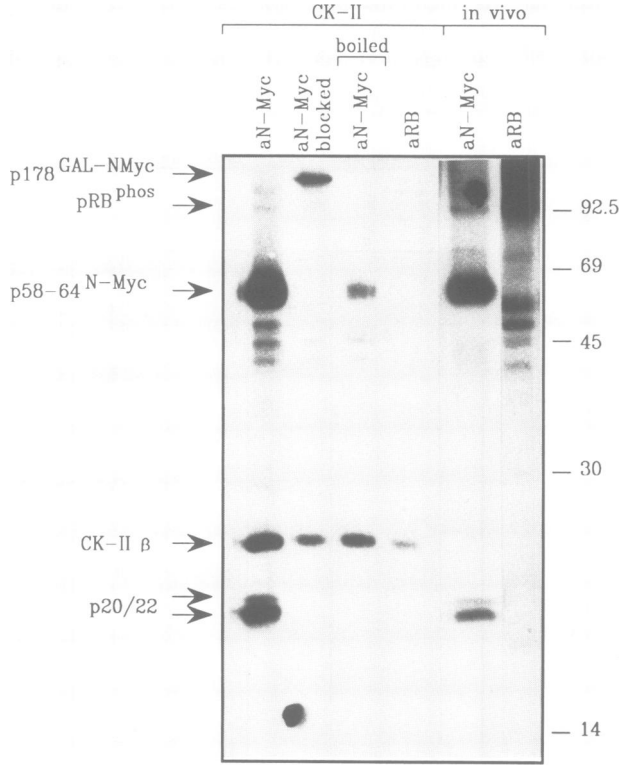

Fig. 4. p20/22 is phosphorylated by CK-II. Extracts from unlabelled Kelly cells were immunoprecipitated with aN-Myc monoclonal antibody and *in vitro* phosphorylated with CK-II. *In vitro* CK-II phosphorylated immunoprecipitations using either blocked aN-Myc or aRB (anti-retinoblastoma gene product) monoclonal antibody were used as controls. As an additional control a boiled extract was precipitated with aN-Myc and also phosphorylated by CK-II *in vitro*. To compare *in vitro* and *in vivo* phosphorylation, extracts from *in vivo* [^{32}P]orthophosphate-labelled Kelly cells were precipitated with aN-Myc and aRB monoclonal antibodies. pRB^{phos} are *in vivo* phosphorylated RB protein species, p178$^{GAL\text{-}NMyc}$ represents the *in vitro* phosphorylated β-galactosidase−N-Myc fusion protein, which was used for blocking of the aN-Myc monoclonal antibody, and CK-II β represents the autophosphorylated β-subunit of CK-II (Lüscher *et al.*, 1989). Proteins were separated on parallel 12.5% SDS−polyacrylamide gels. Size markers are in kDa.

also *in vitro* phosphorylated by CK-II. The *in vitro* phosphorylated proteins were separated on SDS−polyacrylamide gels, the bands corresponding to p22, p20 and p21Max were excised and digested using *Staphyloccocus aureus* V8 protease. The resulting peptides were then separated by polyacrylamide gel electrophoresis and the gels were autoradiographed (Figure 5). Under the experimental conditions used, N-Myc is completely digested (Figure 5, lane 1 and 2) but p22, p20 and Max are all remarkably resistant to proteolysis and a considerable amount of each protein is undigested (Figure 5, lanes 3−8). A similar protease resistance of all three proteins was also observed using chymotrypsin although N-Myc was completely fragmented (data not shown). Comparison of the peptide maps obtained for the three proteins show that p20 and Max give essentially identical peptide patterns with bands differing only in intensity. Two of the major bands of p22 also overlap with p20 and Max. There is a doublet at ~14 kDa from p22 with the higher M_r fragment of the doublet being most intense while the lower band of the doublet with weaker intensity comigrates with the most intense 14 kDa band of p20 and Max. We interpret these results as follows: the resistance of all three proteins to proteases is circumstantial evidence of relatedness. Secondly, we conclude that p20 and Max are cognate homologs while p22 is also related to both p20 and Max with the predominance of the upper band of the 14 kDa doublet being accounted for by the higher M_r of the protein compared with p20 and Max resulting in the generation of a larger fragment.

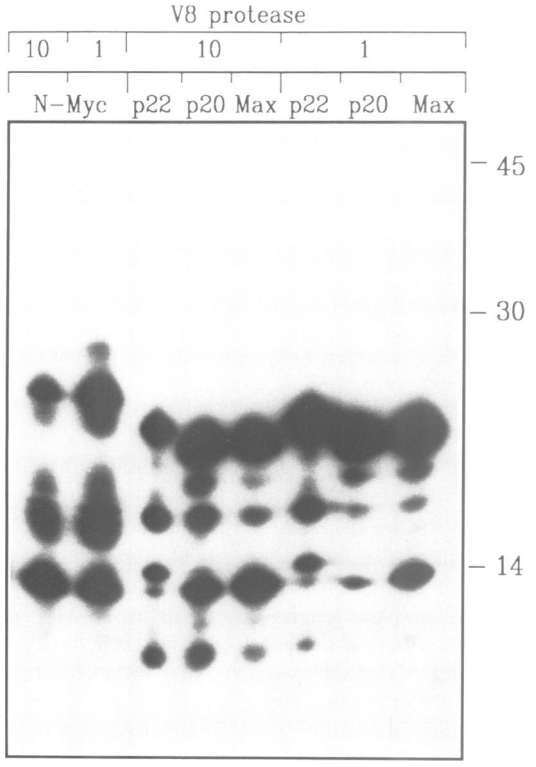

Fig. 5. Comparative peptide analysis of p22, p20 and p21Max. The N-Myc protein as well as p20 and p22 were obtained through immunoprecipitation of extracts prepared from non-labelled Kelly cells and *in vitro* phosphorylated with CK-II. p21Max (Max) was obtained through *in vitro* transcription/translation of pVZ1p21Max. To form *in vitro* N-Myc−Max complexes, aliquots of separately performed *in vitro* translation mixtures containing either p21Max or p58-64$^{N\text{-}Myc}$ were mixed 1:1 in a buffer consisting of one part NTN and NETN. Complexes were formed by incubation on ice for 4 h. The N-Myc−Max complex was immunoprecipitated using the aN-Myc monoclonal antibody and after that *in vitro* phosphorylated with CK-II. Labelled proteins were separated by SDS−PAGE, detected by autoradiography and excised. Digests with two different amounts (given in μg/reaction) of *S.aureus* V8 protease were performed. Peptides were fractionated on a 15% SDS−polyacrylamide gel and exposed for 2 h. Size markers are in kDa.

Blackwood and Eisenman (1991) have recently reported the isolation of using the polymerase chain reaction of additional clones that are identical with Max (151 amino acids) except for a 9 amino acid insertion at the amino terminus resulting in a protein with 160 amino acids. It appears likely that p22 identified by us *in vivo* is the product of this larger mRNA species. To avoid nomenclature confusion we will subsequently use p20/22 for Max as well as for Myn, the murine homolog of Max (Prendergast *et al.*, 1991). Comparison of the respective molecular weights on parallel lanes of SDS−polyacrylamide gels revealed that p20, Max and murine Myn have identical electrophoretic mobility (data not shown).

p20/22 are present in cells of different embryonal lineages

We analyzed whether expression of p20/22 is restricted to neuroectodermal tumor cells carrying N-*myc* amplification or if it is also detectable in cells of different embryonal lineages. As we lack an antibody to analyze p20/22 directly, we employed an indirect assay based on the ability of p20/22 to bind to the N-Myc protein. Various *in vitro* established cell lines were transfected with the N-Myc expression vector pNMYC^{hu}, which we constructed by combining the human N-*myc* gene with a CMV (cytomegalovirus) promoter. Cells were metabolically labelled with ^{32}P, extracted and N-Myc was immunoprecipitated. The autoradiography demonstrates the presence of p20/22 in all cell lines tested, including NIH 3T3 murine fibroblasts, Neuro-2a mouse neuroblastoma cells, human HeLa cells as well as WAC2 cells derived from human SH-EP neuroblastoma cells stably transfected with a N-*myc* expression vector (Figure 6). These results suggest that p20/22 is expressed in a large spectrum of different cell types. Moreover these results also show that the human N-Myc protein forms a complex with heterologous p20/22 expressed in murine cells and indicate that the HLH-Zip domain of Myn(p20/22) responsible for interaction with p58-64$^{N\text{-}Myc}$ is highly conserved.

We have also performed *in vitro* complex formation experiments (Dyson *et al.*, 1989) by mixing extracts from metabolically labelled cells (SH-EP, Neuro-2a, human fibroblasts) expressing only basal amounts of N-Myc protein with extracts from unlabelled cells expressing high amounts of N-Myc protein due to amplification (Kelly) or transiently transfected (Neuro-2a) with the expression vector pNMYC^{hu}. After incubation of the mixtures for a prolonged time (for instance 4−6 h) we found binding of labelled p20/22 to non-labelled p58-64$^{N\text{-}Myc}$ using the aN-Myc mAb in immunoprecipitations (data not shown). The results shown in Figure 6 and the data obtained after *in vitro* complex formation suggest that expression of p20/22 is not induced by N-Myc.

p20/22 interacts with the HLH-Zip domain of N-Myc

We have identified the region in the N-Myc protein responsible for hetero-oligomerization with p20/22. Firstly

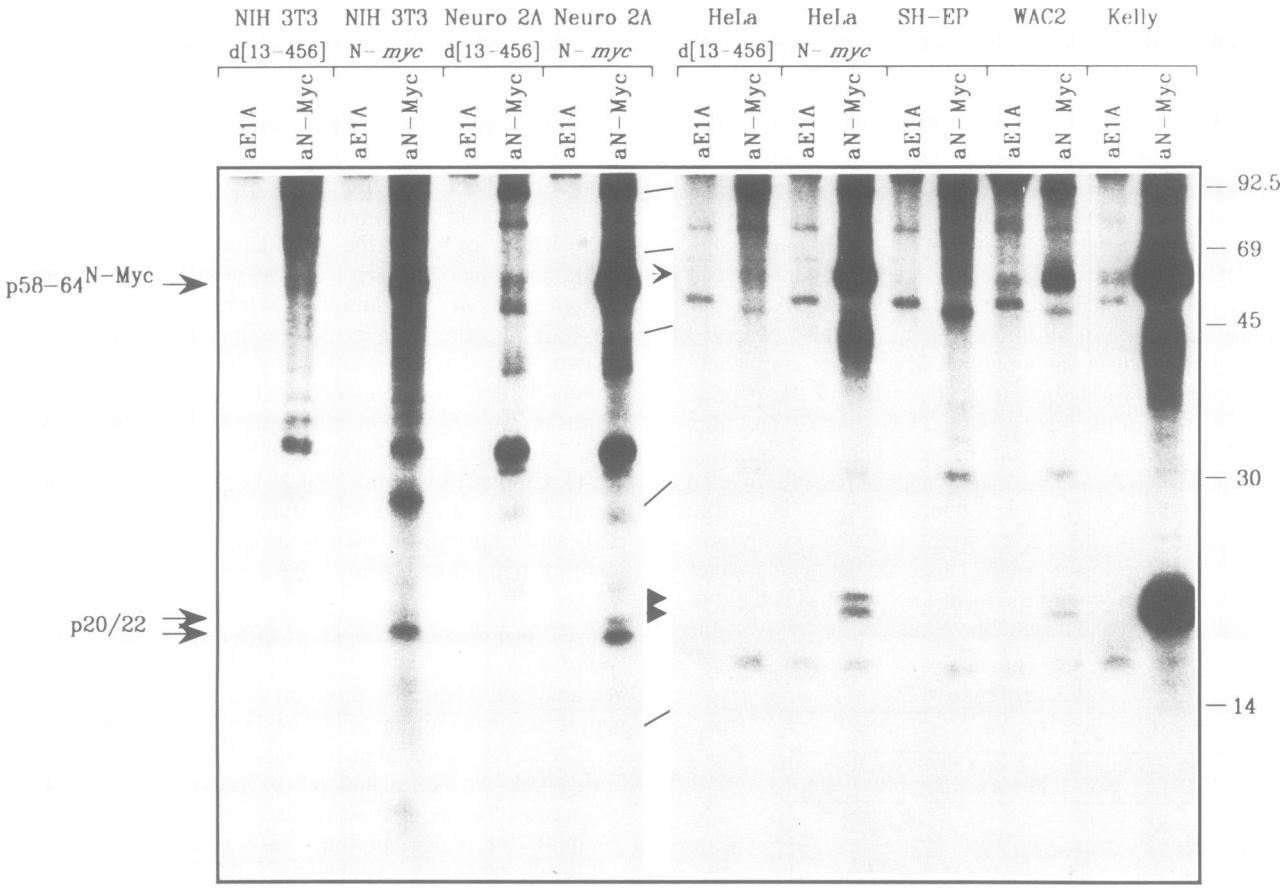

Fig. 6. Expression of p20/22 in different cell types. Mouse NIH 3T3, Neuro-2a, and human HeLa cells transfected transiently with pNMYC^{hu} and WAC2 cells which are derived from human neuroblastoma cell line SH-EP through stable transfection with the N-*myc* expression vector pMP34.1^{k} were analyzed. Immunoprecipitations were performed from extracts of [^{32}P]orthophosphate-labelled cells using the aN-Myc monoclonal antibody. Cells transfected with plasmids coding for a frameshift mutant d[13−456] (see also Figure 7A) and precipitations with aE1A monoclonal antibody served as controls. To compare the relative amounts of immunoprecipitable p58-64$^{N\text{-}Myc}$−p20/22 complex, Kelly cells were used as a reference. Proteins were separated on parallel 12.5% SDS−polyacrylamide gels. Size markers are in kDa.

we generated, by site directed mutagenesis and linker insertions, mutant N-*myc* genes in the expression vector pNMYC^{hu}, that encode N-Myc proteins with in frame and terminal deletions or single amino acid substitutions (Figure 7A). The expression vectors were introduced transiently into mouse Neuro-2a neuroblastoma cells. By Western blot and immunofluorescence analysis we verified that all mutants are expressed and that the proteins are localized in the nucleus of the cell (data not shown). Extracts from transfected and ^{32}P-labelled Neuro-2a cells were used for immunoprecipitation with either aN-Myc or aE1A mAb as a control. Two C-terminal deletion mutants d[387−456] and d[351−456] had lost the capacity to bind p20/22 (Figure 7B, compare lanes b and d with p). In the protein with the smaller deletion d[387−456] the basic region (BR) and part of helix 1 from the HLH domain are still expressed (Figure 7B, lane b). In contrast, internal deletion of the BR and the C-terminal part of the helix 1 in the mutant d[351−387] or the internal deletion as in mutant d[232−269] did not impair the ability to bind p20/22 (Figure 7B, lane f and h). This demonstrates that the site for complex formation is in the C-terminal portion of the N-Myc protein. The importance of an intact leucine zipper domain as well as an intact helix 2 is emphasized by the fact that the exchange of a leucine to a proline at position 396 (L396-P) showed no effect whereas the exchange at position 425 (L425-P) led to a total loss of binding activity (Figure 7B, compare lanes j and l). A single amino acid substitution of leucine 425 for proline causing an α-helical breakage at a site critical for the α-helical conformation of helix 2 in the HLH domain and of the leucine zipper completely abolishes the formation of the N-Myc−p20/22 complex. This indicates that helix 2 and the leucine zipper in the N-Myc protein represent an α-helical domain ('expanded leucine zipper' or 'H-Zip domain') which is responsible for interaction with p20/22. Additional point mutations in the HLH-Zip region will be employed in future experiments to define the region of N-Myc−p20/22 interaction in even greater detail.

Discussion

The C-terminal region of N-Myc like that of c-Myc has features found in other proteins such as transcription factors that are indicative of the potential for dimerization with other cellular proteins. Until now, no data have been reported that would substantiate an association through the HLH-Zip domain of N-Myc, or of the protein encoded by another *myc* family member, in extracts directly prepared from cells. We think that the experimental setting we have used offers a particularly favourable situation for the identification of a N-Myc associated protein since the neuroblastoma cells

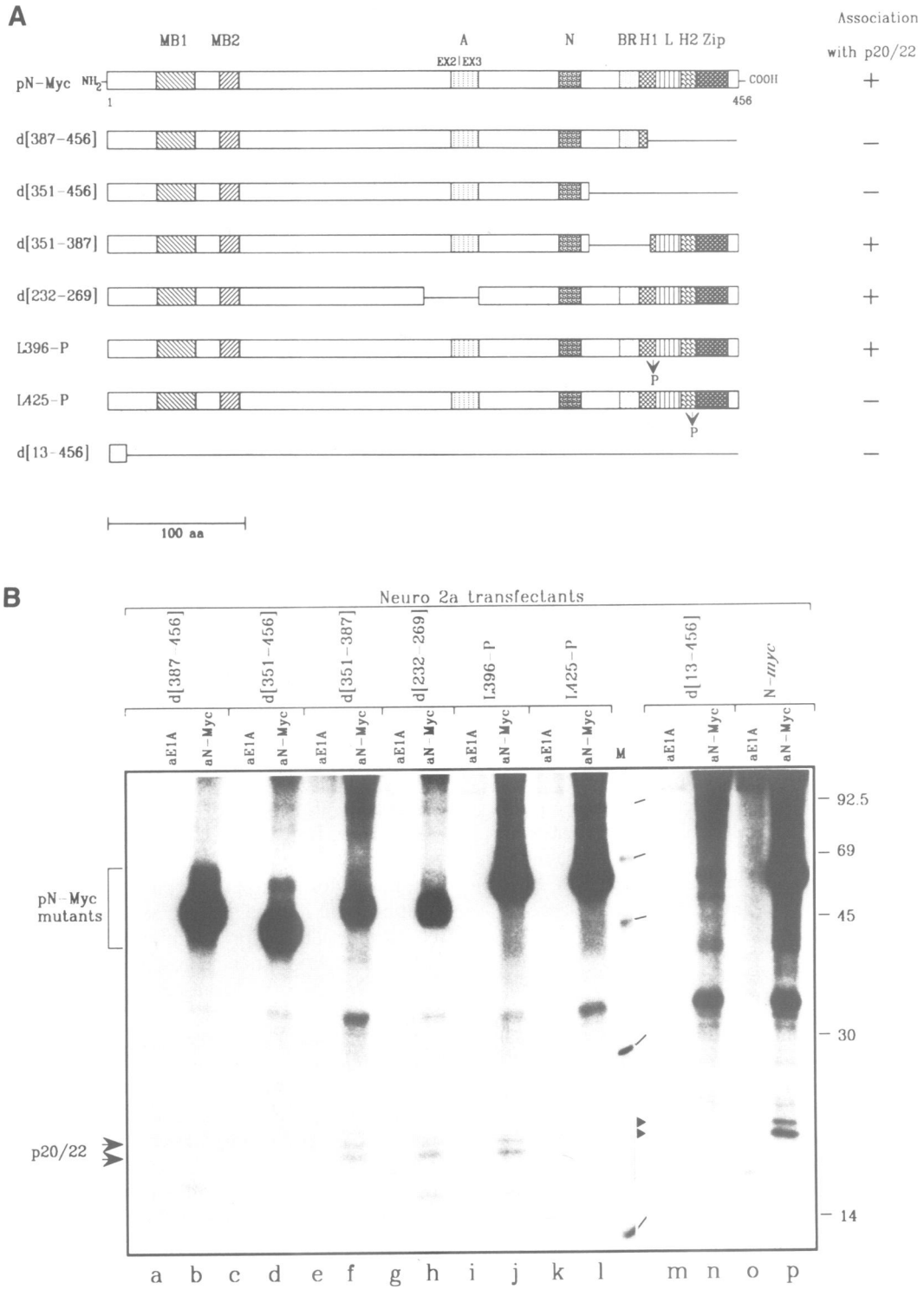

Fig. 7. Interaction of N-Myc and p20/22 is mediated by the carboxy-terminal helix−loop−helix/leucine zipper domain of the N-Myc protein. **A**, Structure of the wild type and mutant N-Myc proteins expressed after transfection as shown in Figure 7B, based on the deduced sequence of 456 amino acids (Stanton *et al.*, 1986). A summary of the results shown in Figure 7B after transfection with regard to the formation of the N-Myc−p20/22 complex is shown on the left (+, association with p20/22; −, no association with p20/22). Deletion mutants (d) are termed d[387−456] (Figure 7B, lanes a, b), d[351−456] (Figure 7B, lanes c, d), d[351−387] (Figure 7B, lanes e, f), d[232−269] (Figure 7B, lanes g, h), d[13−456] (Figure 7B, lanes m, n) with respect to amino acids deleted and point mutations (exchange of leucine [L] against proline [P]) as L396-P (Figure 7B, lanes i, j) and L425-P (Figure 7B, lanes k, l). Important regions in the wild type N-Myc (pN-Myc) and mutant N-Myc protein are shown and termed MB1/MB2 for Myc-Box1 and 2 (Schwab, 1986), A for acidic region at the exon 2−3 border (Ex2, Ex3), N for nuclear localization region (Dang and Lee, 1989), BR for basic region, H1−L−H2 for helix1−loop−helix2 and Zip for leucine zipper. **B**, Extracts from [^{32}P]orthophosphate-labelled mouse Neuro-2a neuroblastoma cells transiently transfected (48 h) and expressing either mutant (lanes a−n) or wild type human N-*myc* (lanes o, p) as described in Figure 7A were immunoprecipitated by using aN-Myc and as a control, aE1A monoclonal antibody. The weaker signals of mutant N-Myc proteins represented by d[351−387] (lane f) and d[232−269] (lane h) are due to deletion of casein kinase II phosphorylation sites located within these deletions (Hamann *et al.*, 1991). Proteins were separated on parallel 12.5% SDS−polyacrylamide gels. Size markers (M) are in kDa.

employed have greatly elevated levels of N-Myc protein as the consequence of N-*myc* amplification.

In this study, we have shown by immunoprecipitation of the N-Myc protein, that two nuclear phosphoproteins, p20/22, can associate with the N-Myc oncoprotein via the helix−loop−helix/leucine zipper region. By partial proteolytic mapping we demonstrated that p20 is identical with the Max protein, a recently identified *in vitro* dimerization partner of myc proteins (Blackwood and Eisenman, 1991) and furthermore that the p22 is a Max-related protein possibly encoded by a clone identical with Max but having an 9 amino acid insertion at its amino terminus which is probably generated by alternative splicing (Blackwood and Eisenman, 1991; Cole, 1991). The association of proteins containing oligomerization motifs such as HLH, Zip or HLH-Zip domains is limited to partners containing homologous domains. The Max protein was shown to contain also a BR-HLH-Zip domain. Max dimerizes with all myc family proteins and c-Myc−Max complexes bind to DNA through the consensus nucleotide sequence CACGTG (Blackwood and Eisenman, 1991). Furthermore these authors also observed that the HLH-Zip region in both proteins is responsible for dimer formation. Because deletion of either c-Myc helix 1 or the leucine zipper or substitution of a helix-disrupting proline residue for the second leucine in the zipper inhibited binding of Max to c-Myc *in vitro*. Prendergast *et al.* (1991) found that deletions into the Zip motif of either c-Myc or Myn are sufficient to eliminate c-Myc−Myn interactions *in vitro*. Our results confirm that the interaction of N-Myc with p20 and p22 is mediated by helix 2 and the leucine zipper. Beyond this our data strongly suggest that these interactions also take place *in vivo* in the cell based on our transfection experiments with mutant N-*myc* genes and subsequent detection of the resulting complexes by immunoprecipitation of cellular extracts. Most likely helix 2 and the leucine zipper represent a large α-helical domain ('expanded leucine zipper' or 'H-Zip domain') which is responsible for the coiled-coil interaction of myc proteins with p20/22. The signal intensities make it likely that less p22 is bound to N-Myc compared with p20 pointing to a difference in binding affinity. If p22 is identical with Max except for a 9 amino acid insertion at the amino terminus as described by Blackwood and Eisenman (1991) then these additional amino acids and (or) their potential post-translational modification by phosphorylation might influence binding affinity. Another possible explanation for the observed heterogeneity of the p20/22 doublet might be that these two proteins are expressed at different levels. Therefore, it is possible that the N-Myc−p20 and N-Myc−p22 complexes have different biological functions especially in those cells where N-Myc is overexpressed.

The N-Myc−p20/22 complex is detectable in cellular extracts even when the immunoprecipitation is performed within 5 min after preparation of the extracts. This observation strongly suggests although it does not formally prove that the complex exists *in vivo* in the cell rather than forming artefactually during immunoprecipitation. In contrast to the data presented by Blackwood and Eisenman (1991) or Prendergast *et al.* (1991) which are based solely on *in vitro* observations, our data reinforce the view that N-Myc−p20/22 complexes are of biological significance.

At this point we can only speculate concerning the biological function of the N-Myc−p20/22 complex. Our results show that p20/22 expression is widespread in contrast to N-Myc, which has a restricted cell type and tissue distribution (Schwab, 1985). The question therefore arises as to which protein might interact with p20/22 in cells lacking N-Myc expression. It may be predicted that any p20/22 interacting protein will have homology to the HLH-Zip region of N-Myc, and it is well possible that in such cells proteins encoded by other *myc* family genes can undergo complex formation with p20/22. If so, it remains to be seen whether there are regulatory mechanisms determining complex formation in cells that co-express different members of the *myc* gene family, such as neuroblastoma cells without N-*myc* amplification that show expression of both N-*myc* and c-*myc* (Breit and Schwab, 1989).

Phosphorylation and dephosphorylation of proteins by protein kinases and phosphatases, respectively, are major cellular mechanisms for regulation and modification of the activity of proteins (Hunter and Cooper, 1985; Edelman *et al.*, 1987). The transmission of gene regulatory signals from the cell surface to the nucleus where regulatory proteins act gene proximal, is mediated by the signal transduction pathway, of which protein kinases are important components (Chen-Wu *et al.*, 1988; Cantley *et al.*, 1991). CK-II is a kinase responsible for phosphorylation of an N-terminal site near the DNA binding domain of the protein encoded by the *myb* proto-oncogene and thereby reversibly inhibits DNA binding of the Myb protein (Lüscher *et al.*, 1990). It also appears that CK-II is involved in the control of cell proliferation (Chen-Wu *et al.*, 1988; Bohmann 1990). Both p20 and p22 are phosphorylated by CK-II *in vitro* as has been shown for the N-Myc and Myc protein which are additionally also phosphorylated by CK-II *in vivo* (Lüscher *et al.*, 1989; Hamann *et al.*, 1991). It remains to be shown whether p20/22 are also substrates for CK-II *in vivo*. The significance of Max(p20/22) phosphorylation is unclear at this time, and it remains to be seen if phosphorylation is a regulatory pathway for determinating complex formation.

An important question not resolved here is the contribution of the N-Myc−p20/22 complex to the development and/or progression of neuroblastoma or other tumors of neuroectodermal origin in which N-*myc* is expressed. In these tumors, of which human neuroblastoma represents a paradigm, the N-*myc* proto-oncogene is activated by amplification (Schwab, 1985). High expression of the gene appears to contribute to progression of this tumor (Schwab *et al.*, 1984). Due to overexpression, the N-Myc protein could act as a scavenger, eliminating p20/22, which could be a negative growth modulator acting in a putative regulatory pathway, and by this route contribute to tumorigenesis. It is also possible that overexpression of N-Myc yields more active N-Myc−p20/22 complexes, a model in which p20/22 could be considered a positive growth modulator. These questions should now be available to investigation using the experimental approaches described here.

Materials and methods

Cell culture and DNA transfection

Human and mouse cell lines were grown in RPMI 1640 with 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% fetal calf serum. Human neuroblastoma cell lines Kelly were provided by Fred Gilbert, NGP, NMB by Garret M.Brodeur and SH-EP by June Biedler. WAC2 cells are derived from human neuroblastoma cell line SH-EP through stable

transfection with the N-*myc* expression vector pMP34.1[k] as previously described (Schwab *et al.*, 1985; Schweigerer *et al.*, 1990). WAC2 cells were grown in the same medium as described above supplemented with 400 ng/ml geniticin sulfate. Mouse neuroblastoma cell line Neuro-2a, NIH 3T3 mouse fibroblasts, human HeLa and 293 cells were obtained from ATCC (USA). Transient transfections with non-mutated or mutated N-*myc* expressed in pNMYC[hu] were performed using standard calcium phosphate–DNA co-precipitations as described by Wigler *et al.* (1977).

Antibodies

The aN-Myc monoclonal antibody (anti-N-Myc; IgG_{2a}) was prepared by using bacterially expressed β-galactosidase–N-Myc fusion protein expressed in pUR 291 (Rüther and Müller-Hill, 1983) as an antigen. Proteolysis products of the fusion protein containing N-terminal parts of N-Myc were purified by SDS–PAGE and used for immunization of Balb/c mice. By subsequent fusion of mouse spleen cells with NSO myeloma cells (Köhler and Milstein, 1975) hybridomas were obtained. Screening of hybridoma supernatants was performed by ELISA (Evan, 1984), and a series of monoclonal antibodies with specificity for the N-Myc protein was selected (unpublished results). Binding of monoclonal antibodies to N-terminal epitopes of N-Myc was verified by Western blot analysis of N-terminal deletion mutants with wild type N-*myc* expressed in pNMYC[hu] (see also the chapter: *In vitro* mutagenesis). Purification of aN-Myc was done with protein G–Sepharose Fast Flow (Pharmacia). The aE1A (anti-adenovirus type 2 and 5 E1A polypeptides; Ab-1; IgG_{2a}) monoclonal antibody as well as rabbit anti-mouse IgG were obtained from Dianova (Hamburg, Germany) and the aRB (anti-retinoblastoma gene product; MAb-1; IgG_1) monoclonal antibody from Triton Sciences (Alameda, USA).

Cell labelling and immunoprecipitations

[^{35}S]methionine labelling of cells and immunoprecipitations were done as described by DeCaprio *et al.*, (1988) with the following modifications: Subconfluent culture dishes of exponentially growing cells were starved for 1 h in methionine-free RPMI 1640 (Biochrom, Berlin) containing 5% dialyzed fetal calf serum. The medium was replaced and supplemented with 300 μCi/ml L[^{35}S]methionine (Amersham-Buchler) and the cells were labelled for 4 h at 37°C. About 2×10^6 cells were lysed in 1 ml of ice-cold NTN [120 mM NaCl, 50 mM Tris–HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM PMSF] and nuclei extracted for 20 min on ice. The lysates were cleared of non-lysed cells, nuclei and debris by centrifugation at 16000 *g* and 4°C for 3×5 min. Resulting supernatants are referred to as extracts. The extracts were precleared by incubation with 100 μl of 50% (w/v) protein A–Sepharose CL-4B (PAS) (Pharmacia) pre-swollen and equilibrated in NETN [100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM PMSF] for 3×10 min on ice. Boiled extracts were prepared by adjusting the extracts to 0.5% SDS and subsequent heating at 100°C for 5 min. Aliquots of either extracts or boiled extracts were diluted 1:1 with NETN, 1 μg of monoclonal antibody was added and the mixture was incubated with head over head agitation for 1 h on ice. Immunocomplexes were collected by adding 30 μl of PAS (for preparation see above) and incubated for additional 30 min. The PAS beads were washed several times in ice-cold NETN. Immunoprecipitated proteins were solubilized in sample buffer and separated by SDS–PAGE.

For labelling with [^{32}P]orthophosphate, exponentially growing cells were starved for 4 h in phosphate-free DMEM (Gibco) containing 5% dialyzed fetal calf serum. The medium was replaced and supplemented with 500 μCi/ml of [^{32}P]orthophosphate (Amersham Buchler), and cells were labelled for 4 h at 37°C. Extraction of cells and immunoprecipitations were done as described above with the exceptions that NTN and NETN contained 100 μM sodium orthovanadate and 100 mM NaF for inhibition of endogenous phosphatases and that where aRB (IgG_1) monoclonal antibody was used 2 μg of rabbit anti-mouse IgG was added and incubated for 20 min prior collecting the immunocomplexes with PAS. Nuclei were prepared as described by Gillespie and Eisenman (1989) and processed as described above. Blocking of the aN-Myc monoclonal antibody prior to immunoprecipitation was performed by incubating 1 μg antibody with 5 μg bacterially expressed β-galactosidase–N-Myc fusion protein in NETN. Immunoprecipitated proteins from ~5×10^5 cell equivalents were loaded per lane, analyzed on SDS–polyacrylamide gels (Laemmli 1970) and detected by fluorography (Bonner and Laskey, 1974) or autoradiography.

In vitro translation

To express N-*myc in vitro* the cDNA (a gift of Larry Stanton) was cloned into pGEM3 (Promega) resulting in pGEM3Nmyc. RNA was obtained through transcription from linearized pGEM3Nmyc with T7 RNA polymerase and Max RNA through transcription from linearized pVZ1p21Max with T3 RNA polymerase. The plasmid encoding Max cDNA was generously provided by E.M.Blackwood and R.N.Eisenman. Resulting RNAs were used in translation reactions with nuclease treated rabbit reticulocyte lysates according to the instructions of the manufacturer (Promega). The amount and integrity of the proteins were evaluated by SDS–PAGE of [^{35}S]methionine-labelled products.

In vitro phosphorylation

Proteins were purified by immunoprecipitation. Aliquots of proteins adsorbed to protein A–Sepharose were prepared and used in the *in vitro* casein kinase II assay as described by Lüscher *et al.*, (1989). A preparation of casein kinase II was kindly provided by David Litchfield. *In vitro* phosphorylated proteins were separated by SDS–PAGE and detected by autoradiography.

Partial proteolytic mapping

Proteins were localized by using the autoradiographs as a template, excised and incubated with different amounts of *Staphylococcus aureus* V8 protease (Boehringer Mannheim) using the method of Cleveland *et al.* (1977). To optimize the digest conditions all buffers used in the V8 digests were adjusted to 0.5% SDS. Partially digested protein products were resolved using 15% SDS–polyacrylamide gels.

In vitro mutagenesis

The in-frame deletions of the N-*myc* gene (Stanton *et al.*, 1986) were generated through linker insertions into restriction sites, some of which were generated through *in vitro* mutagenesis applying the *in vitro* mutagenesis system from Amersham Buchler. Point mutations leading to single amino acid exchanges were also generated by *in vitro* mutagenesis (data not shown). Wild type or mutated N-*myc* was expressed after insertion into pUHD10-1 (Deuschle *et al.*, 1989) containing a promoter derived from cytomegalovirus resulting in the recombinant expression vector pNMYC[hu].

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