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Myristylation of pp60$^{c\text{-}src}$ Is Not Required for Complex Formation with Polyomavirus Middle-T Antigen

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Middle-T antigen (middle-T), the transforming gene product of polyomavirus, associates with several cellular tyrosine kinases, such as pp60$^{c\text{-}src}$. Complex formation leads to kinase activation and is essential for cell transformation. Middle-T-associated as well as uncomplexed pp60$^{c\text{-}src}$ is predominantly found in the plasma membrane. We transfected mouse 3T3 fibroblasts with a mutated c-*src* gene (2Ac-*src*), allowing the expression of a protein containing alanine instead of glycine in position 2 of the primary translation product. Contrary to the wild-type c-*src* gene product, pp60$^{c\text{-}src(2A)}$ was not myristylated and accumulated in the cytoplasm instead of being transferred to cellular membranes. The mutant protein was able to associate with middle-T and was activated similarly to the wild-type c-*src* gene product. Both wild-type and 2A mutant protein were membrane associated upon complex formation with middle-T. This finding suggests that the putative carboxy-terminal membrane anchor sequence of middle-T is sufficient to hold middle-T-associated pp60$^{c\text{-}src(2A)}$ in the plasma membrane.

Transformation of cells by polyomavirus depends on the expression of an intact middle tumor antigen (middle-T) in virus-infected cells (reviewed in reference 17). Both middle-T and large-T must be expressed to transform primary cells in culture (13), whereas transfection of established cell lines with a middle-T cDNA is sufficient to transform cells (36). It has been shown that middle-T forms a stable complex with the cellular tyrosine kinase pp60$^{c\text{-}src}$ (11) and additional members of the *src* kinase family (6, 18, 19). A dramatic increase in kinase activity of middle-T-associated pp60$^{c\text{-}src}$ compared with uncomplexed pp60$^{c\text{-}src}$ has been observed in polyomavirus-infected cells (3).

The complex between middle-T and pp60$^{c\text{-}src}$ is associated with the plasma membrane (1, 10). Both middle-T and pp60$^{c\text{-}src}$ carry membrane-targeting sequences directing these proteins to cellular membranes. Middle-T presumably associates with membranes through interaction of its hydrophobic carboxy terminus with the lipid bilayer (5, 14, 25, 34), whereas the membrane association of pp60^{src} depends on an amino-terminal myristate group covalently linked to glycine 1 of the mature protein (4, 30, 33). It is not clear whether the complex between these two proteins is formed in the cytoplasm immediately after synthesis or after both proteins have reached the plasma membrane as individual entities.

We are interested in the role that myristylation of pp60$^{c\text{-}src}$ might play in complex formation with middle-T and have investigated whether nonmyristylated pp60$^{c\text{-}src(2A)}$ still binds to and is activated by middle-T.

3T3 mouse fibroblasts were transfected with either plasmid pSVc-*src* or pSV2Ac-*src* together with the selectable marker pMOneo^{r} (2). The c-*src* plasmids were derived from a pSV vector described previously (20) and encode, respectively, the wild-type (wt) chicken c-*src* (28) or the mutant 2Ac-*src* gene in which the codon for amino acid 2 has been changed from GGG (glycine) to GCA (alanine). G418-resistant clones were screened by analyzing the expression of pp60$^{c\text{-}src}$ with monoclonal antibody (MAb) EC10, which is specific for the chicken c-*src* gene product (26). Figure 1 shows various 3T3 cell lines expressing either the wt or the mutant protein. Expression of pp60$^{c\text{-}src}$ was monitored by immunoprecipitation of metabolically labeled cells (Fig. 1A and B), by an in vitro kinase assay (Fig. 1C), and by immunoblotting (Fig. 1D). Only the wt product was myristylated (Fig. 1B). In contrast to earlier studies performed with chicken cells (32), the specific activity of the wt c-*src* kinase was 5- to 10-fold higher than that of the nonmyristylated mutant protein; the reason for this disagreement is under investigation. All cell clones had a normal morphology, although pp60$^{c\text{-}src}$ was overexpressed 20- to 50-fold when compared with untransfected 3T3 cells, as described previously (31).

Cells expressing the wt or 2Ac-*src* gene were fractionated into a soluble, cytoplasmic fraction and a postnuclear, particulate fraction containing cellular membranes. The fractionation of pp60$^{c\text{-}src}$ was monitored by in vitro kinase assays (Fig. 2A) and by Western blotting (immunoblotting) (Fig. 2B). In transfected 3T3 cells, wt pp60$^{c\text{-}src}$ was predominantly membrane-associated, whereas the mutant protein was mostly cytoplasmic.

Cells expressing the wt or 2Ac-*src* gene product were infected with either polyomavirus or a retrovirus containing a middle-T cDNA (Py MLV; 15). 3T3 cells expressing middle-T upon retrovirus infection were morphologically transformed and formed foci on a solid substratum but were unable to grow in soft agar, in agreement with earlier findings (29; data not shown). Complex formation between middle-T and the transfected chicken c-*src* protein was observed in MAb EC10 immunoprecipitates derived from transfected 3T3 cells (Fig. 3A). Mutant and wt pp60$^{c\text{-}src}$ were equally efficient in forming this complex, and in both cases middle-T became phosphorylated at the usual site, as determined by *Staphylococcus aureus* V8 protease mapping (Fig. 3B).

Cells transfected with the chicken c-*src* gene and infected with Py MLV were fractionated into a cytoplasmic and a membrane fraction. In wt as well as mutant c-*src*-expressing 3T3 cells, the middle-T/pp60$^{c\text{-}src}$ complex was found exclusively in the membrane fraction (Fig. 3A, lanes 10, 12, 14, and 16). The bulk of the c-*src* gene product was not associated with middle-T and was membrane bound in wt c-*src*-expressing cells (lane 10) but soluble in 2Ac-*src*-transfected cells (lane 13). No middle-T was found in the soluble fraction in either cell line (lanes 9, 11, 13, and 15).

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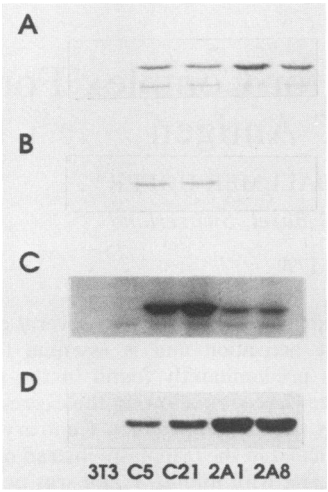

FIG. 1. Expression of pp60$^{c\text{-}src}$ and pp60$^{c\text{-}src(2A)}$ in 3T3 cells. (A) A total of 10^6 cells labeled with [^{35}S]methionine were lysed and immunoprecipitated with MAb EC10; (B) 10^6 cells labeled with [^{3}H]myristic acid were lysed and immunoprecipitated with MAb EC10; (C) in vitro kinase assays were performed with MAb EC10 immunoprecipitates made from 10^6 cells; (D) 2×10^5 cells were lysed in sample buffer and analyzed by immunoblotting with MAb 327 (23). C5 and C21 are representative 3T3 clones expressing wt pp60$^{c\text{-}src}$; 2A1 and 2A8 are clones expressing nonmyristylated pp60$^{c\text{-}src(2A)}$.

In addition to the 56- and 60-kilodalton (kDa) phosphoproteins representing middle-T and pp60$^{c\text{-}src}$, respectively, three bands with molecular sizes of 52, 62, and 85 kDa were observed (Fig. 3A). The 85-kDa band represents a phosphatidylinositol kinase shown to be associated with the pp60$^{c\text{-}src}$/middle-T complex (17). *S. aureus* V8 protease maps of the 56-, 60-, and 62-kDa bands are shown in Fig. 3B. The 56-kDa middle-T band gave rise to two major 24- and 18-kDa fragments as described previously (Fig. 3B, lanes f, g, i, and j; 1). pp60$^{c\text{-}src}$ gave a 26-kDa fragment identified as the carboxy-terminal phosphopeptide, in agreement with earlier reports (lanes b, c, e, and h; 8). The 62-kDa band was not related to pp60$^{c\text{-}src}$ or middle-T (Fig. 3A and Fig. 3B, lanes a and d), whereas the 52-kDa protein represents a truncated form of pp60$^{c\text{-}src}$ that lacks an amino-terminal 10-kDa fragment, as described previously for pp60$^{v\text{-}src}$ (data not shown; 21, 37). Anti-T serum specific for the three early gene products of polyomavirus precipitated more middle-T than did MAb EC10. This finding reflects the fact that middle-T is complexed to the artificially introduced chicken c-*src* gene product as well as the endogenous mouse c-*src*, c-*yes*, and c-*fyn* proteins absent in MAb EC10 immunoprecipitates (Fig. 3A; compare lane 10 with lane 12 and lane 14 with lane 16).

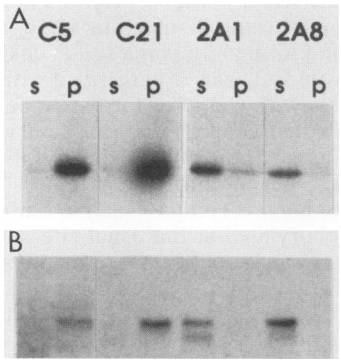

FIG. 2. Membrane localization of pp60$^{c\text{-}src}$ and pp60$^{c\text{-}src(2A)}$. (A) In vitro kinase assays of soluble (s) and membrane (p) fractions derived from transfected 3T3 cells performed with MAb EC10 immunoprecipitates. C5 and C21 are 3T3 cell clones expressing wt pp60$^{c\text{-}src}$; 2A1 and 2A8 are clones expressing pp60$^{c\text{-}src(2A)}$. (B) Immunoblot of cytoplasmic and membrane fractions of transfected 3T3 cells.

Complex formation between middle-T and pp60$^{c\text{-}src}$ is accompanied by dramatic stimulation of the c-*src* kinase activity (3). Figure 4 shows in vitro phosphorylation assays with enolase as an exogenous substrate (9). All polyomavirus-infected cells (Fig. 4B) had greater tyrosine kinase activity than did their uninfected counterparts (Fig. 4A). The activation of wt and mutant chicken pp60$^{c\text{-}src}$ by middle-T was lower than that of the endogenous mammalian c-*src* gene product in nontransfected polyomavirus-infected 3T3 cells; i.e., the relative increase in kinase activity in virus-infected cells versus noninfected 3T3 cells was higher than in virus-infected C5 (wt c-*src*-expressing) and 2A6 (2Ac-*src*-expressing) cells versus noninfected C5 and 2A6 cells (Fig. 4). This result suggests that (i) the mammalian pp60$^{c\text{-}src}$ forms the complex more efficiently than does the transfected chicken homolog or (ii) overexpression of pp60$^{c\text{-}src}$ may influence the efficiency of complex formation between these proteins, perhaps because some cofactors become limiting. No kinase activity was observed in MAb EC10 immunoprecipitates derived from control or polyomavirus-infected 3T3 cells (Fig. 4), confirming that the material immunoprecipitated in C5 and 2A6 cells is derived from the chicken c-*src* gene. In polyomavirus-infected C5 and 2A6 cells, MAb EC10 also recognized the activated c-*src* kinase associated with middle-T (Fig. 4).

In this study, we show that myristylation and membrane localization of pp60$^{c\text{-}src}$ are not required for complex formation with polyomavirus middle-T. In agreement with studies performed with similar c-*src* mutants (30, 32), we found that nonmyristylated pp60$^{c\text{-}src(2A)}$ accumulates in the cytoplasm of the cell. No middle-T/pp60$^{c\text{-}src}$ complex was found in the cytoplasmic fraction, suggesting that the putative membrane anchor of middle-T is sufficient to hold the complex in the plasma membrane. This result can be interpreted in several ways: (i) complex formation occurs in the cytoplasm, followed by rapid transfer to cellular membranes; (ii) middle-T associates with the membrane, where it binds to soluble pp60$^{c\text{-}src(2A)}$, which is then retained in the membrane fraction; or (iii) a small subpopulation of pp60$^{c\text{-}src(2A)}$ (as, for example, in Fig. 2) binds to cellular membranes in a way not requiring amino-terminal myristylation. Association with membrane-bound middle-T may then lead to stable complex formation between these proteins. It was shown earlier that truncated forms of middle-T lacking a functional carboxy terminus are nontransforming (5, 25, 34). A recently described cytoplasmic middle-T mutant consisting of the first 335 amino acids (10) forms a soluble complex with pp60$^{c\text{-}src}$, suggesting that the amino-terminal myristate group present on wt pp60$^{c\text{-}src}$ is not sufficient to direct this mutant middle-T/pp60$^{c\text{-}src}$ complex to the plasma membrane. On the other hand, a mutant lacking only the last 37 amino acids including the hydrophobic carboxy terminus does not form a complex with pp60$^{c\text{-}src}$, presumably because this protein is not properly folded (5, 34). Replacement of the putative middle-T membrane anchor by a similar sequence derived from the vesicular stomatitis virus G protein gave rise to a protein that

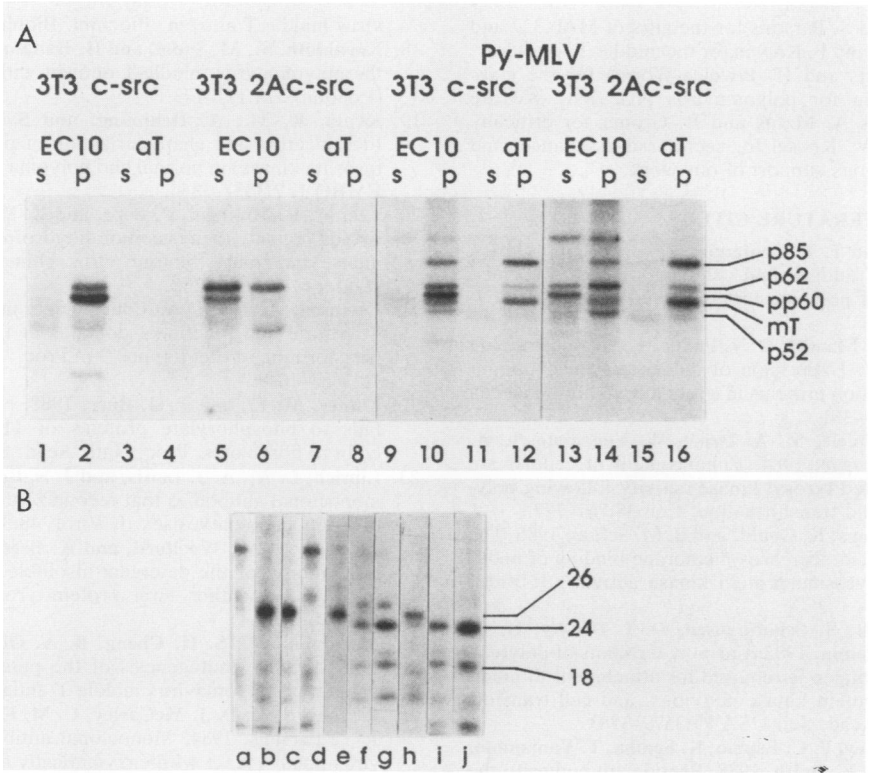

FIG. 3. Localization of the middle-T/pp60$^{c\text{-}src}$ complex in transfected 3T3 cells. (A) Soluble (s) and membrane (p) fractions of pp60$^{c\text{-}src}$, pp60$^{c\text{-}src(2A)}$, and middle-T were analyzed after immunoprecipitation with MAb EC10 (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or anti-T ascites fluid (lanes 3, 4, 7, 8, 11, 12, 15, and 16) in an in vitro kinase assay. The same cell clones are shown uninfected (lanes 1 to 8) or after Py MLV infection (lanes 9 to 16). The bands are identified in the margin. mT, Middle-T. (B) Bands from panel A were excised and digested with *S. aureus* V8 protease as described previously (7). Lanes: a, 62-kDa band from lane 2; b, pp60$^{c\text{-}src}$ from lane 2; c, pp60$^{c\text{-}src(2A)}$ from lane 5; d, 62-kDa band from lane 6; e, pp60$^{c\text{-}src}$ from lane 10; f, middle-T from lane 10; g, middle-T from lane 12; h, pp60$^{c\text{-}src(2A)}$ from lane 13; i, middle-T from lane 14; j, middle-T from lane 16.

formed a complex with pp60$^{c\text{-}src}$ but failed to transform cells (35). Therefore, the association of middle-T with pp60$^{c\text{-}src}$ as well as membrane localization of the complex are essential but not sufficient for cell transformation.

Both wt and 2A mutant pp60$^{c\text{-}src}$ were activated upon complex formation with middle-T (Fig. 4). The relative increase in pp60$^{c\text{-}src}$ kinase activity was more pronounced in control 3T3 cells than in transfected cells. This is probably a consequence of the overexpression of pp60$^{c\text{-}src}$ in these cells or a reduction in the ability of the chicken gene product to associate with middle-T. Furthermore, the transfected chicken c-*src* protein may be partially activated as a result of the high level of expression even in the absence of middle-T.

The finding that the 2A mutant protein is not able to associate with cellular membranes is reminiscent of earlier studies performed with pp60$^{v\text{-}src}$ (4, 12, 33). Several mutants of pp60$^{v\text{-}src}$ have been described that give rise to a nonmyristylated gene product although they retain the amino-terminal glycine moiety (12, 27). In general, v-*src* mutants not capable of associating with the plasma membrane and the cytoskeleton are nontransforming (16, 24). A few mutants isolated from recovered avian retroviruses are transforming although they are not membrane associated, presumably because they still bind tightly to cytoskeletal structures (16). This finding suggests that increased pp60^{src} kinase activity is not sufficient for cell transformation. Additional factors, such as specific localization of the protein in the cell leading to phosphorylation of new substrates localized perhaps in the cytoskeleton, seem to be important for cell transformation (22).

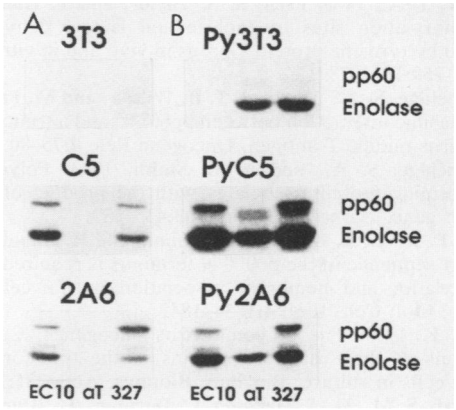

FIG. 4. Activation of pp60$^{c\text{-}src}$ and pp60$^{c\text{-}src(2A)}$ by middle-T antigen. Gels show phosphorylation of enolase in an in vitro kinase assay after immunoprecipitation of various mock-infected (A) or polyomavirus NG59RA (Py)-infected 3T3 (1) cell lines (B) with MAb EC10, anti-T ascites fluid (aT), or MAb 327 as indicated at the bottom. The lysates were corrected for equal protein content before immunoprecipitation; the antibody was present in excess. The infection rates were measured as the percentage of T-antigen-positive cells determined by immunofluorescence with anti-T serum (3T3 cells, 60%; wt c-*src*-expressing 3T3 cells, 30%; 2Ac-*src*-expressing 3T3 cells, 16%). Gels were exposed for 6 h (3T3 and C5 cells) or 16 h (2A6 cells) with an intensifying screen on Kodak XAR-5 film at −70°C.

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