The β-Amyloid Precursor Protein APP Is Tyrosine-phosphorylated in Cells Expressing a Constitutively Active Form of the Abl Protooncogene*

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The cytosolic domain of the β-amyloid precursor protein APP interacts with three PTB (phosphotyrosine binding domain)-containing adaptor proteins, Fe65, X11, and mDab1. Through these adaptors, other molecules can be recruited at the cytodynamic of APP; one of them is Mena, that binds to the WW domain (a protein module with two conserved tryptophans) of Fe65. The enabled and disabled genes of Drosophila, homologues of the mammalian Mena and mDab1 genes, respectively, are genetic modulators of the phenotype observed in flies null for the Abl tyrosine kinase gene. The involvement of Mena and mDab1 in the APP- centered protein-protein interaction network suggests the possibility that Abl plays a role in APP biology. We show that Fe65, through its WW domain, binds in vitro and in vivo the active form of Abl. Furthermore, in cells expressing the active form of Abl, APP is tyrosine-phosphorylated. Phosphopeptide analysis and site-directed mutagenesis support the hypothesis that Tyr^682 of APP_695 is the target of this phosphorylation. Co-immunoprecipitation experiments demonstrate that active Abl and tyrosine-phosphorylated APP also form a stable complex, which could result from the interaction of the pYENP motif of the APP cytodynamic with the SH2 domain of Abl. These results suggest that Abl, Mena, and mDab1 are involved in a common molecular machinery that and APP can play a role in tyrosine kinase-mediated signaling.

The amyloid plaques, the major pathological hallmark of Alzheimer's disease (AD), contain the β-amyloid peptide, which results from the proteolytic cleavage of a membrane protein known as β-amyloid precursor protein (APP) (for review see Ref. 1). The functions of APP are still poorly understood, despite a significant amount of information concerning its involvement in a complex protein-protein interaction network. APP is a type I membrane protein with a small cytosolic domain that has been shown to interact with several soluble proteins. Three of these proteins, Fe65, X11, and mDab1, interact with APP through a PTB domain (2–8). They have the characteristics of adaptor proteins, and thus they could connect APP to different intracellular molecular pathways. In addition to the PTB domain, X11 has two PDZ (Pez-DigAl/ZO-1) domains and forms oligomeric complexes with other two proteins, Munc-18 and CASK (9, 10). Fe65 possesses two PTB domains (PTB1 and PTB2), one of which interacts with APP (5) and the other of which interacts with LRP (low density lipoprotein receptor-related protein), a scavenger receptor structurally related to the low density lipoprotein receptor (11), and with the transcription factor CP2/LSF/LBP1 (12). At the N terminus, Fe65 possesses a WW domain, through which it forms complexes with several proteins including Mena (13), the mammalian homologue of the product of the Drosophila enabled gene. The third PTB-containing protein that interacts with APP is mDab1 (7, 8), the mammalian homologue of the product of the Drosophila disabled gene. Genetic studies have demonstrated that insects bearing the mutation of the gene homologous to the mammalian c-abl tyrosine kinase gene survive past morphogenesis with some defects in eye structure, but when this mutation is associated with heterozygous or homozygous mutations of the disabled gene, defects in the formation of central nervous system are observed. Insects that are homozygous for mutations in both Abl and disabled genes show an almost complete loss of proper axonal connections (14–16). These defects are significantly ameliorated by mutations of the enabled gene (17, 18). The functions of the corresponding mammalian proteins, Mena and mDab1, are probably similar. In fact, mice lacking mDab1 show severe abnormalities in the development of the central nervous system, including defects in migration of neurons (19), whereas the mutation of Mena causes defects in neurulation and commissure formation (20).

The c-abl proto-oncogene is the cellular counterpart of the viral oncogene of the Abelson murine leukemia virus, and its gene is involved in the Philadelphia chromosome translocation (9;22) that generates the BCR-Abl fusion protein in human leukemia (for review see Ref. 21). Abl is a nonreceptor tyrosine kinase similar to c-Src. At the N-terminal half, Abl contains an SH3 and an SH2 domain, and in the middle it contains a kinase...
domain. In contrast to Src and other kinases, its C terminus is long and contains nuclear localization, nuclear export signals, and domains that are able to interact with DNA and F-actin (for recent reviews, see Refs. 22 and 23). The intracellular distribution of Abl is very peculiar; among the various isoforms of Abl, one (type IV) is membrane-associated and another is soluble (type I). The latter can be found in either the cytosol or the nucleus. The cytosolic Abl interacts with the actin cytoskeleton (24), whereas in the nucleus it can bind DNA and other proteins (25).

Despite the large amount of experimental data on Abl, its functions are still not fully understood. In particular the molecular mechanisms connecting Drosophila Abl to disabled and enabled and mammalian Abl to mDab1 and Mena are not known. However, the opposite effects of the mutations of the enabled and disabled genes in Drosophila suggest that the products of these genes may be involved in the same molecular pathway. This conjecture is in agreement with the observations that both mDab1 and Mena participate in the protein-protein interaction network centered at the cytodomain of APP and supports the hypothesis that c-Abl also could be involved in the APP-centered molecular machinery. In this report we demonstrate that APP is tyrosine-phosphorylated in cells expressing a constitutively active form of Abl. Active Abl is tightly connected to the oligomeric complexes formed by APP, because Abl binds to the WW domain of Fe65 and is co-immunoprecipitated with APP.

**EXPERIMENTAL PROCEDURES**

**Recombinant Constructs**—The Fe65 and APP95 expression constructs have been described (5). Mouse c-Abl type IV cDNA, cloned into the pCDNA3 vector (Invitrogen), was a kind gift of A. Costanzo. Abl-PP expressing the vector and pGEX-Abl-SH2 (26) were kindly provided by D. Barila and G. Superti-Furga. To generate the APPY653F, Y682F, APP-695 expression vector and pGEX-Abl-SH2 (26) were kindly provided by D. Barila and G. Superti-Furga. To generate the APPY653F, Y682F, APP-695 expression vector and pGEX-Abl-SH2 (26) were kindly provided by D. Barila and G. Superti-Furga. The recombinant constructs were sequenced by the dideoxy termination method with the Sequenase kit (Amersham Pharmacia Biotech) to confirm the inserted mutations. All plasmids were propagated following standard procedures (27) and purified on Qiagen Maxi-columns (Qiagen).

**Cell Culture, Transfections, and Extract Preparation**—CO2 atmosphere. For transfection, 3 green monkey kidney cells were cultured in Dulbecco’s modified minimal essential medium (Gibco-BRL) according to manufacturer’s instructions, with the following six primer pairs (Cengia): 5’-GGGTAAGTGGCAAGAGAAGAGAGTCACAGCCTACCTCTCCTATGTGATTGGG-3’; 5’-CCACCGCACTCATGATGAATGGATGTGCACACCTGGCATAATCTCAAGATGCAGCAGAGCAG-3’; 5’-GATTCTGCTGACAGGCAAGAAGAGAGTCACAGCCTACCTCTCCTATGTGATTGGG-3’; 5’-CCACCGCACTCATGATGAATGGATGTGCACACCTGGCATAATCTCAAGATGCAGCAGAGCAG-3’; 5’-CCACCGCACTCATGATGAATGGATGTGCACACCTGGCATAATCTCAAGATGCAGCAGAGCAG-3’; 5’-CCACCGCACTCATGATGAATGGATGTGCACACCTGGCATAATCTCAAGATGCAGCAGAGCAG-3’. Amplification of the inserts was performed in the absence of any protein source and in the presence of nonspecific immune complex. The reaction products were fractionated onto 12.5% SDS-PAGE, stained with Coomassie R-250 (Bio-Rad), and exposed to autoradiographic films. For phosphopeptide analysis (30), the reaction products were resolved by SDS-PAGE and transferred to the electrode. Tyrosine Phosphorylation of APP by Abl

**RESULTS**

**Abl Interacts with the WW Domain of the Fe65 Adaptor Protein**—It was recently hypothesized that Abl could interact with APP through the adaptor proteins, which are known to bind to the APP cytodomain (32). We explored the possibility that Fe65, one of the modular proteins that forms complexes with APP, could be one of the adaptors that tethers Abl to the cytodomain of APP. To address this point we performed pull-down experiments in which GST-Fe65 fusion proteins were used as baits to entrap Abl from extracts of CO2 cells transfected with plasmid vectors driving the expression of type IV mouse c-Abl or of Abl-PP, in which the mutation of two prolines of c-Abl (Pro242 and Pro249), located between the SH2 domain and the TK domain, results in a constitutive TK activity (33). As shown in Fig. 1A, both PTB domains of Fe65 do not interact with Abl-PP. On the contrary, Abl-PP is affinity-purified by the GST-Fe65 fusion protein containing the WW domain. A similar result was observed by using the extracts from cells transfected with c-Abl, which similarly interacts only with the WW domain of the Fe65 protein.
sequence of Abl, there is at least one motif that can be recognized by the WW domain of Fe65 (PPPXXXPPPP) located between amino acids 899 and 904 (13, 34).

The Cytosolic Domain of APP Is Tyr-phosphorylated in Cells Expressing a Constitutively Active Form of Abl—The Fe65-Abl-PP interaction supports the hypothesis that Fe65 functions as a docking site to bring Abl close to phosphorylation targets. To evaluate whether Fe65 itself is a target of Abl TK, the same approach was undertaken. Abl-PP interaction supports the hypothesis that Fe65 functions as a docking site to bring Abl close to phosphorylation targets. To evaluate whether Fe65 itself is a target of Abl TK, the same approach was undertaken.

Fig. 1. Fe65 and active Abl interact through the WW domain of Fe65. A, glutathione-Sepharose beads were saturated with wild type GST (lanes 2 and 7) or with recombinant GST-Fe65 fusion proteins containing the PTB1 (lane 3), PTB2 (lane 4), or WW (lanes 5 and 6) domains of Fe65 and were incubated with extracts from COS7 cells co-transfected with Abl-PP (lanes 1–5) or c-Abl (lanes 6–8) expression vectors. Bound proteins were eluted from the resin and resolved by 8% SDS-PAGE. Western blot (WB) was with Abl antibody. The bottom panels (lanes 2–5, 7, and 8) show the Ponceau S staining of the filters that indicates the amount of each GST protein used. Lanes 1 and 6 contain 10 μg of the lysates used in the pull-down experiments. B, protein extracts from COS7 cells transfected with either Abl-PP (lanes 1–6) or mouse type IV c-Abl (lanes 7–9) expression vectors were immunoprecipitated (IP) with Fe65 antibody or, as a control, with preimmune serum (PI) and resolved by 8% SDS-PAGE. Western blot (lanes 1, 2, and 7–9) was with Abl antibody. The same filter of lanes 1 and 2 was reprobed with anti-pTyr antibody (lanes 3 and 4) and then with Fe65 antibody (lanes 5 and 6). The arrowhead indicates the Abl band and the asterisk the Fe65 bands.

To evaluate whether the band immunoprecipitated by the APP antibody and stained by the anti-pTyr antibody is the consequence of a Tyr phosphorylation of the cytosolic domain, a peptide was synthesized on the basis of the C-terminal sequence of APP, common to all the APP isoforms. This peptide was incubated in the presence of [γ-32P]ATP with an Abl-immunoprecipitated extract of COS7 cells expressing Abl-PP. Fig. 2B shows the autoradiography of the SDS-PAGE gel and the peptide result to be 32P-labeled only when incubated with the proteins immunoprecipitated with the Abl antibody and not with the proteins immunoprecipitated with unrelated antibodies. The labeled band was transferred onto a polyvinylidene difluoride filter, and the peptide was hydrolyzed as described under “Experimental Procedures.” The resulting amino acids were separated by thin layer chromatography, and 32P-labeled amino acids were detected by autoradiography. Fig. 2C shows that the only 32P-labeled residue found in the hydrolyzed peptide was phosphotyrosine.

There are three tyrosines located in the cytoplasmic domain of APP (residues 653, 682, and 687 of APP695). To analyze their involvement in the above described phenomena, we generated three mutant APP695 expression vectors in which Tyr653 or Tyr682 has been changed into phenylalanine or Tyr687 has been changed into alanine. These vectors were transfected in COS7 cells with the Abl-PP-expressing vector. As shown in Fig. 2D, the APP-Y653F and APP-Y687A are recognized by the anti-pTyr antibody, whereas the APP-Y682F is not; this suggests the hypothesis that Tyr682 is the actual target of the phosphorylation of the citodomain of APP. This result is in agreement with the data concerning the substrate preference of protein tyrosine kinases, indicating that c-Abl preferentially phosphorylates proteins at the level of the YXXP motif (37), where X are hydrophilic residues, which fits with the YENP sequence we found to be phosphorylated in APP.

APP Co-immunoprecipitates with the Active Form of Abl—The Western blot experiment of Fig. 2A (see lane 2), in addition to the APP bands also shows a slower migrating band recognized by the anti-pTyr antibody and co-immunoprecipitated by the APP antibody. This band is present only in the samples from COS7 cells co-transfected with Abl-PP, and its size is compatible with that of Abl itself. This possibility was analyzed in the experiments reported in Fig. 3; panel A shows the Western blot analysis with Abl antibody of proteins immunoprecipitated by APP antibody that demonstrated the presence of Abl in the immunoprecipitates. The co-immunoprecipitation of Abl with APP has been observed also in cells not transfected with APP (see Fig. 3A, lane 3), thus suggesting that the transfected, active Abl could also be co-immunoprecipitated with endogenous APP770, present in significant amounts in COS7 cells. The co-immunoprecipitation of APP and Abl was confirmed in the experiment reported in Fig. 3B, in which protein extracts from COS7 cells were immunoprecipitated with Abl antibody and blotted with APP. Also in this case APP is present in the extract immunoprecipitated by the Abl antibody.

It was demonstrated that the various known protein tyrosine kinases phosphorylate Tyr-containing sites that, upon phosphorylation, are recognized by their own or closely related SH2 domains of Abl to phosphorylate APP in vivo.
Tyrosine Phosphorylation of APP by Abl

**fig. 2. APP is tyrosine-phosphorylated in cells expressing the active form of Abl.** A, extracts from COS7 cells transfected with Abl-PP and/or APP$_{695}$ expression vectors (as indicated) were immunoprecipitated (IP) with anti-APP 369 (lanes 1–4) or anti-pTyr (lanes 6 and 7) antibodies and analyzed by Western blot (WB) with anti-pTyr (lanes 1 and 2) or anti-APP 369 (lanes 3–7) antibodies. Lanes 3 and 4 refer to the same filter as in lanes 1 and 2 stripped and reblotted with APP antibody. Lane 5 contains 10 μg of extract from cells transfected with Abl-PP and APP expression vectors. B, a 38-amino acid-long peptide designed on the basis of the extreme C-terminal sequence common to all of the APP isoforms was incubated in the presence of [γ-$^32$P]ATP with immunoprecipitated proteins from COS7 cells transfected with Abl-PP expression vector. Abl antibody was used for immunoprecipitation, and mouse IgG were used as a control. Reaction mixtures were separated by 12.5% SDS-PAGE. Lanes 1–4 show the Coomassie staining of the gel, and lanes 2′–4′ show the autoradiography of the same gel. Lanes 2 and 2′, peptide incubated with [γ-$^32$P]ATP; lanes 3 and 3′, peptide incubated with [γ-$^32$P]ATP and mouse IgG-immunoprecipitated proteins; lanes 4 and 4′, peptide incubated with [γ-$^32$P]ATP and anti-Abl-immunoprecipitated proteins. The arrowhead indicates the migration of peptide band. C, the $^32$P-labeled peptide was hydrolyzed, and the resulting amino acids were separated by thin layer chromatography as described under “Experimental Procedures.” The migration of the phospho-amino acid standard is reported: pTyr; pThr, phosphothreonine; and pSer, phosphoserine. D, extracts from COS7 cells transfected with Abl-PP and with wild type APP$_{695}$ (lanes 1 and 5) or mutants APP Y653F (lanes 2 and 6), APP Y682F (lanes 3 and 7), or APP Y687A (lanes 4 and 8) expression vectors were immunoprecipitated with anti-APP antibodies 4G8 and 6E10 and analyzed by Western blot with anti-pTyr antibody (lanes 1–4). The same filter was reprobed with anti-APP 369 antibody (lanes 5–8).

**Discussion**

We addressed the question of the possible involvement of Abl in the protein-protein interaction network centered at the cytosolic domain of APP and found that APP is tyrosine-phosphorylated in cells expressing a constitutively active Abl. In addition we showed that this protein forms complexes with Fe65 and with APP itself. Our results suggest that Fe65 could bind at the same time APP, through its PTB2 domain, and Abl, through its WW domain, thus allowing the formation of a heterotrimeric complex. As a consequence of this interaction, active Abl is docked close to the APP cytodomain, and this could favor the phosphorylation of its Tyr$^{682}$ (see a hypothetical model in Fig. 4). The observed co-immunoprecipitation of Tyr-phosphorylated APP and Abl could be the consequence of APP-Abl interaction through the SH2 domain of Abl, which has a high affinity for the pYXXP motif (38). Furthermore, the phosphorylation of Tyr$^{682}$ is expected to be deleterious for the binding of the PTB domains of Fe65, X11, and mDab1 because it was demonstrated that, at least for Fe65 and X11, this residue is crucial for the formation of the complexes with APP (3), and it is a hydrophobic residue in most of the known PTB binding sites (39).

As reported in Fig. 4, there are several lines of evidence suggesting that Abl, mDab1, and Mena are involved in common molecular machineries. It was, in fact, demonstrated that mDab1 binds through its PTB domain with the cytosolic domain of APP (7, 8) and that Mena interacts with the WW domain of Fe65 (13), which, in turn, interacts with APP. Considering the results reported in this paper, it can be hypothesized that Abl, mDab1, and Mena compete for the anchoring to the same intracellular site: mDab1 by competing with Fe65 for the binding to the cytodomain of APP; and Abl and Mena by
and the possible correlation with the defects caused by DAbl, disabled, and enabled gene mutations is not apparent. However, one could gain better insight by the analysis of the phenotypes of insects bearing combined mutations of Appl with the other three genes. For example, the effects of disabled gene mutation on the Abd–/– flies also could be the consequence of the direct interaction of these two proteins with Appl, whereas the amelioration observed in Drosophila Abd–/– disabled–/– following the mutation of the enabled gene could be also based on the competition between the enabled and DAbl gene products for the binding to Appl through Drosophila Fe65.

Although the WW domain of Fe65 interacts in vitro with both c-Abl and Abl-PP, only the complexes between Fe65 and the active form of Abl, and not those with the wild type c-Abl, were found in cell extracts. This effect could be due to a lower amount of c-Abl than Abl-PP available for the formation of the in vivo complexes; or it could be due to a low affinity of c-Abl for the WW domain of Fe65 because of the competition with other ligands of the WW domain of this protein. Furthermore, active Abl probably has a different conformation than c-Abl, thus acquiring a higher affinity for the WW domain. On the contrary, the APP-Abl direct interaction probably requires an active Abl, because the binding is based on a pTyr-SH2 interaction.

It has been hypothesized often that APP could have some role in signaling, and in a recent review article, Bothwell and Giniger (32) suggested the possibility that intracellular signaling could be involved in the development of AD. Their hypothesis takes into account the numerous reports on various proteins that could be involved in the pathogenesis of AD and suggests a role for c-Abl as a modulator of APP biology. Our results support their hypothesis. A point that deserves attention, besides those discussed in the mentioned review article, concerns the possible involvement of p73 in the molecular machinery under examination. In fact, this protein is a key regulator of apoptosis that binds to and is activated by Abl as

![Diagram](Image URL)
a response to DNA damage (40–42). An isoform of p73 functions as an anti-apoptotic protein in developing neurons (43), and the role of its phosphorylation by Abl has not been addressed. The finding that active Abl binds to APP suggested an examination of the possible regulatory effects of this binding on the p73 phosphorylation by Abl and the consequences on this regulation of the enhanced APP proteolytic processing characteristic of AD.

Our results support the hypothesis that Fe65 connects Abl to the APP-centered molecular machinery. However, other possible roles of the Fe65-Abl complex should be examined, as for example those suggested by the observation that both Fe65 and Abl, further than in the cytoplasm, are also localized in the nucleus (24, 44).

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