Two Distinct Intracytoplasmic Regions of the T-cell Adhesion Molecule CD28 Participate in Phosphatidylinositol 3-Kinase Association*

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Through the interaction with its ligands, CD80/B7-1 and CD86/B7-2 or B70, the human CD28 molecule plays a major functional role as a costimulator of T cells along with the CD3TcR complex. We and others have previously reported that phosphatidylinositol 3-kinase inducibly associates with CD28. This association is mediated by the SH2 domains of the p85 adaptor subunit interacting with a cytoplasmic YMNM consensus motif present in CD28 at position 173–176. Disruption of this binding site by site-directed mutagenesis abolishes CD28-induced activation events in a murine T-cell hybridoma transfected with human CD28 gene.

Here we show that the last 10 residues of the intracytoplasmic domain of CD28 (residues 193–202) are required for its costimulatory function. These residues are involved in interleukin-2 secretion, p85 binding, and CD28-associated phosphatidylinositol 3-kinase activity. In contrast, the CD28/C8D8 interaction is unaffected by this deletion, as is the induction of other second messengers such as the rise in intracellular calcium and tyrosine phosphorylation of CD28-specific substrates. Furthermore, we also demonstrate that, within these residues, the tyrosine at position 200 is involved in p85 binding, probably together with the short proline-rich motif present between residues 190 and 194 (PYAPP).

In the absence of a costimulatory signal, activation of the CD3TcR complex is not sufficient to induce the complete activation of T lymphocytes. The interaction between CD28 on T lymphocytes and its counter-receptors CD80 (B7-1) and CD86 (B70 or B7-2) on antigen-presenting cells provides a costimulatory signal required for IL-2 production, T-cell proliferation, and effector functions such as T-cell-mediated cytotoxicity and differentiation of Th cells into Th1 or Th2 subsets (for recent reviews, see Refs. 1–3). This CD28/CD80 interaction has also been shown to prevent anergy and to boost anti-tumor immunity (4–6).

Sequence comparisons between human, rat, mouse, and chicken CD28 cytoplasmic domains (7–10) demonstrate high interspecies conservation, suggesting a crucial role for this domain in coupling to signal transduction pathways. In the absence of catalytic motifs in this sequence, an indirect coupling via adaptor molecules was the most likely mechanism of action. Indeed, we and others have demonstrated previously that ligand stimulation of the human CD28 molecule induces its association with PI 3-kinase activity (11–15) by means of a cytoplasmic YMNM motif at position 173–176 which, when phosphorylated, interacts with the SH2 domains of the p85 adaptor subunit. Similarly, the SH2 domain of the adaptor protein Grb-2 has been shown to interact with this motif although with a lower affinity (16), and the CD28-associated Grb-2-Sos complexes are likely to link the activated CD28 receptor to the activation of p21ras and downstream events such as Raf-1 hyperphosphorylation and ERK2 stimulation (17), as well as JNK kinase activation (18).

The primary events leading to CD28 phosphorylation are becoming better understood. The T-cell-specific protein-tyrosine kinase ITK has been shown to associate with CD28 and to be phosphorylated on tyrosine residues after CD28 stimulation (19), and the Src-related tyrosine kinases p56lck and p59fyn have been found in CD28 immune complexes from stimulated T cells (20). Recently, it has been shown that CD28 is phosphorylated by p56lck and p59fyn in vitro leading to the recruitment of ITK, Grb-2, and p85 (21). Interestingly, the pattern of tyrosine phosphoproteins induced by a CD28 stimulation is similar but not superimposable to that induced by a CD3TcR stimulation (22, 23) and, among the identified products, are p36–38, p95bav, and PLC-γ1 as well as a CD28-specific 64-kDa protein which has yet to be formally identified (reviewed in Ref. 24).

Using a murine T-cell hybridoma transfected with the human CD28 gene, we have shown previously that a point mutation of the Tyr173 residue into phenylalanine abolished CD28-induced IL-2 secretion, suggesting that the PI 3-kinase pathway plays a major role in the CD28 function (12). Here we report the generation and functional characterization of a set of intracytoplasmic variants of the human CD28 molecule. We have generated mutants of CD28 containing progressive truncations of its intracytoplasmic tail (10, 21, 30, and 41 residues), as well as a point mutation of the tyrosine residue at position 200. These variants were expressed in a murine T-cell hybri-
doma. By analyzing stable transfectants, we investigated whether these molecules were able to mediate cell adhesion to human CD80-transfected L-cells, to be phosphorylated, bind and activate PI 3-kinase, and to costimulate IL-2 production together with CD3-TCR.

**EXPERIMENTAL PROCEDURES**

Cells and mAbs—DC27.1 used for transfection is a murine T-cell hybridoma derived by transfecting the TcR αβ genes of KB,C50 in D011.10.2 (kindly provided by B. Malissen, CIML, France). These cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, sodium pyruvate (111 μM), l-α- methionine (50 μM), and antibiotics (penicillin-streptomycin, 10 IU/ml), supplemented by xanthine (250 μg/ml), hypoxanthine (13.6 μg/ml), and mycophenolic acid (2 μg/ml). LTK− and LB7− cells are L-cells respectively untransfected or transfected by a CD80/B7-containing expression vector. The human CD28 mAbs, CD28.1, CD28.2, CD28.3, CD28.5, CD28.6, and 248 used in this study have been described previously (25). C11E4 and 6A11.2 (anti-human IgG1 and IgM, respectively) were derived in the laboratory and used as negative isotypic controls. The anti-murine mAbs were, respectively, 145-2C11 (a hamster IgG specific for CD3ε chain) and 37.51 (specific for murine CD28, Pharmingen, San Diego, CA).

Oligonucleotide-directed Mutagenesis—The human CD28 cDNA (kind gift of B. Seed, Ref. 7) was cloned into the pBluescript KS (kind gift of B. Seed, Ref. 7) was cloned into the CA).

Plasmid Construction and Transfection—Wild type and mutated CD28 cDNA constructs were cloned into pHAPR-1-neo (26) at Sall/BamHI sites, and recombinant genes were introduced by protoplast fusion into D011.10.2 as described (27). Stable transfectants were selected for their resistance to 3 mg/ml genetin G418 (Life Technologies, Inc.) and antibiotics (penicillin-streptomycin, 10 IU/ml), supplemented by hypoxanthine (13.6 μg/ml), mycophenolic acid (2 μg/ml). LTK− and LB7− cells are L-cells respectively untransfected or transfected by a CD80/B7-containing expression vector. The human CD28 mAbs, CD28.1, CD28.2, CD28.3, CD28.5, CD28.6, and 248 used in this study have been described previously (25). C11E4 and 6A11.2 (anti-human IgG1 and IgM, respectively) were derived in the laboratory and used as negative isotypic controls. The anti-murine mAbs were, respectively, 145-2C11 (a hamster IgG specific for CD3ε chain) and 37.51 (specific for murine CD28, Pharmingen, San Diego, CA).

Flow Cytometry Analysis—2 × 105 cells were incubated with saturating concentrations of mAbs at 4 °C for 1 h. After extensive washing, cells were stained with fluorescein isothiocyanate-conjugated goat anti-mouse Ig at 4°C for 30 min (Jackson Laboratories, West Grove, PA). The samples were analyzed by flow cytometry using a FACScan (Becton Dickinson). Fluorescence data were collected with logarithmic amplification.

Adhesion Assay—4 × 105 transfected cells loaded with calcein AM (Molecular Probes, Eugene, OR) were added to 105 LTK− or LB7− cells seeded at 105 in a microtiter plate in 1% FCS in PBS. Cells in the absence of transfected cells were used as controls. Adherent cells were analyzed by the quantification of fluorescence (excitation at 485 nm and emission at 538 nm) by flow cytometry (Fluorocan).

Measurement of IL-2 Secretion—106 transfected cells were stimulated for 24 h at 37 °C in microtiter plates with various stimuli. 5 × 104 units of IL-2 or 10 ng of IL-1 were added to CD28-transfected (LB7−) L-cells used for stimulation of transfectants. Negative (anti-CD5) and positive (anti-CD28) controls were respectively purified mAbs C11E4 and 145-2C11 used at a final concentration of 10 μg/ml in combination with Frε+ B lymphoma cells LK35.10 (106 cells). Soluble CD28.2 mAb (30 μg/ml) was used in combination with soluble 145-2C11 (10 μg/ml). Culture supernatants were collected and titrated, by serial 2-fold dilutions, for their ability to support proliferation of the IL-2-dependent murine T-cell line, CTLL-2, as assessed by the cell growth determination 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide kit (Sigma). Results were expressed as A570 nm obtained for each dilution of the supernatants.

PI 3-Kinase Assay—105 transfected cells were either unstimulated or activated by CD28.2, then immunoprecipitated with protein G-Sepharose 4B (Pharmacia Biotech Inc.) or with a p85 antisemur (UBI). Measure of CD28-associated PI 3-kinase activity was performed as described in Ref. 12.

Association of p85 Subunit and CD28 Phosphorylation—106 cells were stimulated by a fibroblast cell line transfected (LB7−) or not (LTK−) by the CD80/B7 cDNA, with or without CTLA-4i-g. Western blotting of p85-associated CD28 molecules was performed as described in Ref. 12.

RESULTS

Binding of B7/CD80-transfected Cells and CD28 mAbs to CD28 Intracytoplasmic Deletion Mutants Transfected into a Murine Hybridoma—The high conservation of the CD28 cytoplasmic domain among various species (human, rat, mouse, chicken) suggested its major role in signal transduction. Nonetheless, in the absence of a recognizable catalytic domain within these sequences, an indirect coupling via adaptor molecules was suspected. We truncated 10 (del 10), 21 (del 21), 30 (del 30) C-terminal residues, respectively, or the whole intracellular domain (del 41) (Fig. 1A) and stably transfected these various constructs into the murine T-cell hybridoma DC27.1. Fig. 1B shows CD28 expression profiles for one done representative of each transfection, after staining with the CD28.2 mAb. Deletion of 10, 21, 30, or 41 residues did not prevent surface expression of the transfected molecule (Fig. 1B), but for the del 41 mutant, the mean fluorescence intensity was 7-fold lower than that observed for wild type CD28 (49 and 370, respectively).

We also tested these cells for staining with a panel of 5 distinct mAbs: CD28.1, CD28.4, CD28.5, CD28.6, and 248 identifying at least 4 distinct epitopes on the CD28 molecule (25), as well as for binding of a B7-1 fusion protein, and they all stained the various CD28 deletion mutants (not shown).

CD28 is an adhesion molecule since CD28/CD80 interaction allows cell adhesion (31). Using L cells transfected with human CD80, we show that wild type CD28-expressing cells bound to huCD80+ cells (LB7−, 34.5% of binding) but not to untransfected cells (LTK−). In addition, this binding was inhibited by the addition of the human mAb CD28.2A (Fig. 1C). The del 10 and del 30 transfected mutants were still able to bind huCD80−L cells with almost similar efficacy to wild type CD28. We previously reported the involvement of the tyrosine residue at position 173 in the activation of the PI 3-kinase pathway (12). Fig. 1C shows that this mutation did not affect CD28/CD80 interaction. Altogether, these data indicate that all deleted CD28 molecules still bind CD28 mAbs and B7-1 and, in addition, are equally able to mediate the CD28/CD80 interaction showing that their extracellular structure was not modified.

The 10 C-terminal Residues of CD28 Are Required for IL-2
Secretion—We have previously shown that a point mutation of the tyrosine residue at position 173 abolished both PI3-kinase binding and IL-2 secretion in a murine T-cell hybridoma transfected with human CD28 (12). Here we investigated whether other regions of the CD28 cytoplasmic domain were required for late events of activation. We therefore tested whether CD28 stimulations (CD80-L cells or CD28 mAb in combination with CD3 mAb) could induce IL-2 secretion in transfected cells. Fig. 2 shows that both stimulations induced IL-2 secretion in cells transfected by the wild type CD28 construct (upper panel), while IL-2 production was severely altered in del 10 cells whatever CD28 stimuli was used (lower panel). Similar data were obtained with cells transfected by molecules truncated by 21, 30, and 41 residues (not shown). By contrast, cross-linked CD3 stimulation resulted in strong IL-2 secretion in all these clones.

Coupling of Deleted CD28 Molecules to Phosphatidylinositol 3-Kinase and Tyrosine Kinases—We and others have shown previously that, upon stimulation, the human CD28 molecule was able to associate with a PI 3-kinase activity. This association involves the SH2 domains of the p85 adaptor subunit, p85 Western blotting of CD28 immunoprecipitates revealed that deletion of 10 C-terminal residues decreased the CD28/p85 association by more than 90% while a deletion of 30 C-terminal amino acids including residues 173–176 completely abolished it (Ref. 12 and data not shown).

We also examined the ability of other transducing pathways to associate with CD28 deletion mutants. A rise in Ca2+ reflecting PLCγ1 activation was detected in cells expressing either wild type CD28 or del 10 mutant upon stimulation by CD3, as well as by CD28 mAbs (data not shown). CD28 and CD3 stimulations induce the tyrosine phosphorylation of specific substrates (17, 22, 23). A 2-min stimulation of both WT and del 10 transfected cells by CD3 mAbs induced the tyrosine phosphorylation of several substrates, the two most prominent

**Fig. 1.** Intracytoplasmic truncations of the human CD28 molecule. A, deletion mutants were produced by replacing original codons by stop codons (arrows) using oligonucleotide-directed mutagenesis. Sequencing of mutated molecules before transfection was performed according to the classical dideoxy method. B, one clone representative of each transfection (wild type or deleted CD28 molecules) was analyzed by flow cytometry after staining with the CD28.2 mAb. These fluorescence histograms were compared with staining of the untransfected murine T-cell hybridoma, DC27.1. C, adhesion assay was performed using untransfected (LTK−) or CD80−L cells in the absence (LB7+), or presence of the CD28.2 mAb (LB7+ /CD28.2).

**Fig. 2.** Function of wild type and deleted CD28 molecules. Transfected cells were stimulated by cross-linked CD5 (closed circles) or CD3 (open circles) mAbs as negative and positive controls, respectively. CD28 stimulations were performed with CD80−L cells (triangles), or soluble CD28 mAb in combination with soluble CD3 (closed squares). Supernatants were collected after 24 h of stimulation and titrated by serial dilutions for their ability to support proliferation of the IL-2-dependent cell line, CTLL-2. Results are expressed as A570 nm obtained for each dilution of the supernatants and correspond to the proliferation of CTLL-2 as assessed by the cell growth determination 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. Stimulation by soluble CD28 or CD3 mAbs on their own did not induce a significant IL-2 production.

Here we tested gradually truncated CD28 molecules for their ability to associate with p85 and PI 3-kinase. Upon CD28 mAb stimulation PI 3-kinase activity associated with the wild type CD28 molecule (Fig. 3, lane 2), while a deletion of 30 C-terminal residues including the p85 binding site completely abolished this coupling (lane 8). Interestingly a deletion of only the 10 last residues was also able to inhibit 90% of the PI 3-kinase activity coupled to the CD28 molecule (lane 4). Since the total immunoprecipitable PI 3-kinase activity was equivalent in all these cells (data not shown), the observed defect in PI 3-kinase activity could be explained either by the inability of truncated molecules to activate the enzyme or by their failure to associate with its p85 adaptor subunit. p85
bands corresponding to molecular masses of 100 and 36 kDa (Fig. 4, lanes 2 and 5 (32, 33)). CD28 stimulation led to a strong phosphorylation of two proteins of 95 and 64 kDa, the former being vav. As shown in Fig. 4, deletion of the 10 C-terminal amino acids did not prevent phosphorylation of these substrates.

Binding of CD28 Peptides to p85 C-SH2 and SH3 Domains—The cytoplasmic CD28 sequence contains two short proline-rich segments between residues 178–183 (PxxPxxP) and 190–194 (PxxPP) which might serve as docking sites for the SH3 domain of p85 (34). Deletion of 10 C-terminal residues (amino acids 193–202) disrupts one of these proline-rich sequences, and this may account for the observed defect in p85 binding. To test this hypothesis and to determine if individual SH3 and C-SH2 domains of p85 could bind directly to the C-terminal part of CD28 in vitro, we tested whether a 17-mer peptide corresponding to residues 186–202 of CD28 could interact with recombinant SH2 and SH3 domain fusion proteins. As shown previously, interaction of p85 with the YMMM consensus site was strictly dependent upon tyrosine phosphorylation since a 15-mer phosphopeptide corresponding to residues 166–180 strongly bound the C-SH2 domain of p85 (Fig. 5, lane 4) while a nonphosphorylated form of the peptide did not (lane 2). In a non-phosphorylated form, peptide 186–202 did not interact with the C-SH2 domain (lane 6) while it did bind the p85 SH3 domain (lane 7). Interestingly, this p85 SH3 domain binding was decreased when peptide 186–202 was phosphorylated at position Tyr200 (lane 10). Despite the absence of a consensus p85 binding site in the phosphopeptide 186–202, a weak binding of the p85 C-SH2 domain was observed however (lane 9).

Involvement of Tyr200 in CD28 Signaling—To examine if the tyrosine residue at position 200 was involved in the PI 3-kinase pathway in vivo, we mutated it to phenylalanine and expressed the mutated construct in DC27.1 cells. Stable cell lines were analyzed for surface expression, CD80 binding, IL-2 secretion, p85 binding, and PI 3-kinase activation. Fig. 6 shows that point mutation of Tyr200 → Phe200 inhibited CD28-associated PI 3-kinase activity but did not abolish it (lane 4). Western blotting of p85 demonstrated that this impairment was due to a decrease in the quantity of CD28-associated p85 (not shown).

It has never been proven that PI 3-kinase association with CD28 was necessary for activation of the enzyme. We have therefore tested CD28-induced accumulation of D-3 phosphoinositides in the transfectants expressing wild type or mutated (Tyr173, Tyr200) CD28 molecules. In wild type transfectants, B7 ligation induces a transient accumulation of PtdIns(3,4,5)P3 (Fig. 6B). However, point mutation of the Tyr173 residue completely abolished CD28-induced PI 3-kinase activation as assessed by PtdIns(3,4,5)P3 accumulation. In contrast, a point mutation of the Tyr200 residue, however, only delayed and

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attenuated its activation. Thus, the defect in the PI 3-kinase activation observed in the DYF200 transfectant occurs at the level of p85 association.

We also examined the ability of p85 C-SH2 domain fusion proteins to precipitate wild type or mutated CD28 molecules following ligation. Fig. 7A shows that upon ligation by CD80$^{1}$-L cells, the GST-p85 C-SH2 fusion protein precipitated wild type CD28 (lane 2), while a point mutation of Tyr$^{173}$ strongly decreased this interaction (lane 8). Mutation of Tyr$^{200}$ did not prevent CD28 interaction with p85 C-SH2 domain (lane 5). Mutation of both Tyr$^{173}$ and Tyr$^{200}$ to phenylalanine abrogated most of the CD28 ability to be recognized by the C-SH2 domain of p85 (lane 11).

CD28 is tyrosine-phosphorylated upon activation (12, 21) and mainly on the Tyr$^{173}$ residue (21). Mutation of the Tyr$^{200}$ residue did not prevent CD28 phosphorylation, while point mutation of Tyr$^{173}$ strongly decreased it. The double mutant Tyr$^{173}$–200 lost most of its ability to be tyrosine-phosphorylated after stimulation (Fig. 7B). Hence, CD28 phosphorylation is further decreased by a double point mutation.

The tyrosine residue at position 200 is therefore involved in PI 3-kinase binding and activation. We have also tested its role in CD28 function. Fig. 8 shows that CD28 mAbs in combination with CD3 mAbs induced IL-2 secretion although to a lesser extent than wild type CD28. In contrast, stimulation by CD80$^{1}$-L cells did not induce IL-2 secretion (Fig. 8) while binding to CD80$^{1}$-L cells was retained (not shown).

**DISCUSSION**

In this report, we studied the structural requirements of the cytoplasmic domain of human CD28 for its signaling. For this analysis, the wild type CD28 molecule and various cytoplasmic mutants (deletion of 10, 21, 30, and 41 amino acids, or point mutation of Tyr$^{200}$ → Phe) were expressed into the murine T-cell hybridoma DC27.1. We have shown previously that transfection of the full-length CD28 cDNA in these cells allowed surface expression of functional molecules which induce either early or late events of T-cell activation (27). Flow cytometric analysis showed that deletion of 10, 21, or 30 residues did not affect the cell surface expression of CD28. Deletion of the whole intracellular domain, however, impaired the expression of the construct. This observation has previously been reported for mutational analysis of the human CD2 molecule and could be explained by a partial instability of the molecule due to the removal of positively charged amino acids which are responsible for transmembrane stabilization (35).

After ligand binding and dimerization, many growth factor receptors phosphorylate several substrates on tyrosine residues leading to a cascade of signaling events. The antigen-binding T-cell receptor does not possess intrinsic enzymatic activity, and its coupling to the cellular signaling machinery is mediated by adaptor molecules. Mutagenesis studies of several molecules involved in T-cell functions (CD3 $\zeta$ chain, CD2) have identified cytoplasmic consensus motifs which couple these receptors to early events of T-cell activation. The ITAM motif...
characterization of clones carrying mutations of CD28 confirms that the CD28 cytoplasmic domain plays a major role in signal transduction. We show that deletion of the 10 C-terminal amino acids severely impairs IL-2 secretion induced by a CD28 stimulation. This impairment was not merely due to a modification of CD28 extracellular structure, since all epitopes, recognized by 6 different CD28 mAbs (25) were retained on the various deleted molecules, and since these transfectants were equally able to bind to B7-1g and CD80-transfected L cells. Furthermore, cells carrying a deletion of 10 C-terminal residues were able to exhibit wild type levels of calcium mobilization as well as tyrosine phosphorylation of cellular substrates in response to CD28 stimulation. This suggests that the most C-terminal region of CD28 (residues 193–202) is crucial for the coupling of this receptor to IL-2 secretion. Interestingly, this region of CD28 is also involved in PI 3-kinase binding and activation. Together with the previously described loss of CD28 function following mutations of the PI 3-kinase binding site at residues Tyr173 (12) and Met176 (39), our data argue for the major role of this enzyme and/or its associated molecules in coupling the CD28 receptor to the cellular events leading to IL-2 secretion.

Upon ligand interaction, CD28 becomes tyrosine-phosphorylated and associates with p85 via a 173YMNM motif present in its cytoplasmic domain since a point mutation of Tyr173 completely abolished p85 binding to CD28 (12, 13, 20). Recently, Raab et al. (21) have shown that p56lck and p59fyn can phosphorylate the Tyr173 residue of CD28 in vitro. Interestingly, mutation of this residue did not completely abolish CD28 phosphorylation denoting the presence of other phosphorylation sites (21). Here we show that a deletion of 10 C-terminal residues greatly diminished the ability of CD28 to bind PI 3-kinase without affecting other signaling pathways such as PLCγ activation and tyrosine phosphorylations. Within this region, we have further identified two putative motifs involved in p85 binding. The first is a short proline-rich region (residues 190–194), and the second a tyrosine residue at position 200. We mutated this tyrosine residue (Tyr200) and confirmed its involvement in p85 binding and PI 3-kinase activation in vivo. Interestingly, deletion of the last 10 amino acids and point mutation of Tyr200 only decreased PI 3-kinase binding to CD28. A low, but detectable, amount of the p85 still associated with mutated CD28. Furthermore, in vitro binding experiments showed that while the binding of the p85 SH2 domain to this Tyr200 residue was dependent upon its phosphorylation, it was weak compared to binding to the 173YMNM motif. This observation was not surprising since Tyr200 is not located within a consensus binding site for SH2 domains of p85 (YxxM, Ref. 40). Two alternative non-consensus binding sites, YVXXV (41) and YVNA (42), have also been described as novel p85 recognition motifs in the tyrosine kinase receptors HGF-R and Flt-1, respectively.

The results we present here demonstrate that two regions in the intracytoplasmic domain of CD28 are involved in PI 3-kinase binding, one corresponding to the consensus p85 binding site 173YMNM and another one at the C terminus of the molecule (residues 193 to 202) including tyrosine 200 within a non-consensus p85 binding site. Although the CD28 173YMNM motif is sufficient to associate with p85 since individual N- or C-SH2 fusion proteins can coprecipitate CD28 after CD28-B7 interaction and a 15-mer CD28 peptide including phosphorylated Tyr173 precipitates PI 3-kinase from cell lysate (not shown), we propose that the two SH2 domains of p85 act in concert to associate with two distinct tyrosine residues of CD28 in vivo. The first is present within the consensus sequence 173YMNM and the other at position 200 is a non-consensus...
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