Direct Interaction between the Cytoplasmic Tail of ADAM 12 and the Src Homology 3 Domain of p85α Activates Phosphatidylinositol 3-Kinase in C2C12 Cells*

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ADAM 12, a member of the ADAM family of transmembrane metalloprotease-disintegrins, has been implicated previously in the differentiation of skeletal myoblasts. In the present study, we show that the cytoplasmic tail of mouse ADAM 12 interacts in vitro and in vivo with the Src homology 3 domain of the p85α regulatory subunit of phosphatidylinositol (PI) 3-kinase. By site-directed mutagenesis, we have identified three p85α-binding sites in ADAM 12 involving PXXP motifs located at amino acids 825–828, 833–836, and 884–887. Using green fluorescent protein (GFP)-pleckstrin homology (PH) domain fusion protein as a probe for PI 3-kinase lipid products, we have further demonstrated that expression of ADAM 12 in C2C12 cells resulted in translocation of GFP-PH to the plasma membrane. This suggests that transmembrane ADAM 12, by providing docking sites for the Src homology 3 domain of p85α, activates PI 3-kinase by mediating its recruitment to the membrane. Because PI 3-kinase is critical for terminal differentiation of myoblasts, and because expression of ADAM 12 is up-regulated at the onset of the differentiation process, ADAM 12-mediated activation may constitute one of the regulatory mechanisms for PI 3-kinase during myoblast differentiation.

ADAMs1 (proteins containing a disintegrin and metalloprotease) are a family of transmembrane or secreted glycoproteins that have been implicated in cell surface proteolysis, adhesion, and cell-cell communication (1–3). A typical ADAM protein contains an N-terminal pro-domain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich region, and usually an EGF-like repeat, a single transmembrane domain, and a cytoplasmic tail. ADAM 12 has been implicated in differentiation of skeletal muscle precursor cells (myoblasts) in vitro (4) and in vivo (5–8). ADAM 12 expression has been shown to be dramatically up-regulated during embryonic muscle development (6) and in regeneration of adult muscle following injury (7, 8). The extracellular portion of ADAM 12 contains a zinc-dependent metalloprotease (9) that is negatively regulated by the presence of the pro-domain (10). The cysteine-rich domain (11, 12), disintegrin-like domain (13), or the two domains together (14) have been demonstrated to mediate cell-cell adhesion and communication. The intracellular domain of ADAM 12 has recently been shown to interact with actin cytoskeleton via α-actinin-2 (7) and α-actinin-1.‡ Moreover, the cytoplasmic tail of ADAM 12 contains several Src homology 3 (SH3) binding motifs, and therefore it has been anticipated to interact with SH3 domain-containing proteins. Indeed, it has been recently demonstrated that ADAM 12 binds to the SH3 domain of non-receptor protein kinase Src (15, 16) and to an adapter protein, GRB2 (15). Moreover, interaction with ADAM 12 led to stimulation of the enzymatic activity of Src (16).

Phosphatidylinositol 3-kinase (PI 3-kinase) is essential for terminal differentiation of skeletal muscle cells. Two specific and structurally unrelated inhibitors of PI 3-kinase, LY294002 and wortmannin, block myoblast exit from the cell cycle, inhibit expression of muscle specific genes, and abolish the capacity of myoblasts to form myotubes (17, 18). Expression of dominant-negative mutants of PI 3-kinase inhibits myoblast fusion and biochemical differentiation (18, 19). Moreover, expression of a constitutively active form of PI 3-kinase encoded by a viral oncogene, v-p38, strongly enhances differentiation and fusion of cultured myoblasts, suggesting that the cellular PI 3-kinase constitutes a rate-limiting step of myogenesis in vitro (18).

Insulin growth factors, the well known inducers of myogenic differentiation (20–22) and potent stimulators of myoblast survival (23), have recently been shown to exert their function through activation of the PI 3-kinase-dependent signaling pathways (24, 25). Finally, NF-κB and nitric-oxide synthase, identified as downstream effectors of PI 3-kinase in myoblasts, were shown to be critical for myogenic differentiation (26).

Based on their structures, lipid specificity, and modes of regulation, PI 3-kinases can be divided into three classes (27–29). Class I enzymes are heterodimers composed of an 110-kDa catalytic subunit and a regulatory subunit. Class I can be further divided into subclasses IA and IB, which are regulated by tyrosine kinases and G protein-coupled receptors, respectively. Each of the class IA regulatory subunits contains two SH2 domains that bind to phosphotyrosine residues in activated tyrosine kinase receptors or adaptor proteins and play critical roles in translocation of the cytosolic PI 3-kinases to the plasma membrane. In addition, two of the class IA regulatory subunits, p85α and p85β, contain a single SH3 domain. Importantly, a p85α-associated PI 3-kinase appears to be indispensable for myogenesis (18, 19).

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1 The abbreviations used are: ADAM, protein containing a disintegrin and metalloprotease; aa, amino acid; ARNO, Arf nucleotide binding site opener; SH3, Src homology 3 domain; SH2, Src homology 2 domain; GST, glutathione-S-transferase; CBP, calmodulin-binding peptide; GFP, green fluorescent protein; PH, pleckstrin homology domain; DPBS, Dulbecco’s phosphate-buffered saline; GST, glutathione-S-transferase; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

‡ Y. Cao and A. Zolkiewska, unpublished observation.
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In the present study, we show that the cytoplasmic tail of ADAM 12 interacts with the SH3 domain of the p85α regulatory subunit of PI 3-kinase in vitro and in vivo. By site-directed mutagenesis, we mapped the p85α-binding sites to three different regions in ADAM 12 cytoplasmic tail. Using green fluorescent protein-plekstrin homology (GFP-PH) domain fusion protein as a probe for PI 3-kinase lipid products in the plasma membrane of intact cells, we further demonstrate that the expression of ADAM 12 in C2C12 cells leads to activation of PI 3-kinase. Therefore, interaction of the SH3 domain of p85α with the cytoplasmic domain of ADAM 12 could be a novel mechanism of activation of PI 3-kinase in myoblasts.

**Experimental Procedures**

**Antibodies**—Anti-ADAM 12 antibody has been described previously (16). Mouse monoclonal and rabbit polyclonal anti-p85α antibodies were purchased from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology (Lake Placid, NY), respectively.

**Expression Constructs**—Bacterial expression constructs encoding GST-P1–5, P1–4, P1–3, P1–2, P1–5, calmodulin-binding peptide (CBP)-tagged P1–5, and the cytoplasmic tail of integrin β3 were described previously (16). DNA fragments encoding the P34 region of ADAM 12 (as 505–903) and the CH3 domain of ADAM 12, amplified by polymerase chain reaction (PCR) using mouse skeletal muscle cDNA (CLONTECH, Palo Alto, CA) as template, Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA), and appropriate sets of primers. PCR products were cloned into the pGEX-2T vector (Amersham Pharmacia Biotech) between the BamHI and EcoRI sites for expression of glutathione S-transferase (GST) fusion proteins in *Escherichia coli*. The construction of the full-length ADAM 12 plasmid and ADAM 12-(Δ1–424), which encodes a truncated form of mouse ADAM 12 (aa 425–903) lacking the pro- and metalloprotease domains and containing an exoglycosidase secretion signal, has been described previously (16). To engineer a membrane-targeted ADAM 12 cytoplasmic tail, a myristylation motif corresponding to the first 7 amino acids of mouse c-Src was cloned into a membrane-targeted ADAM 12 cytoplasmic tail, C2C12 cells were lysed with buffer A (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM 4-(2-aminoethyl)-benzene-sulfonylfluoride hydrochloride (AEBSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) using 2 ml buffer/100-mm plate. The cell extract was subjected to centrifugation (15,000 *g*, 20 min), and the supernatant was incubated with glutathione–Sepharose (50 μl/ml lysate) for 1 h. Pre-cleared cell lysate (6 ml) was applied onto column (0.2 ml bed volume) containing GST fusion proteins (0.6 mg). The column was washed with buffer B (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100) and eluted with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 μl/ml phosphatase and 0.1% Triton X-100 (buffer C). To study the interaction of ADAM 12, ADAM-12(Δ1–424), and ADAM-12(Δ1–424) mutants with the SH3 domain of p85α, pre-cleared lysate from transfected COS-7 (1 ml) was applied onto column (0.1 ml of resin) containing GST-SH3 or GST (0.2 mg). The columns were washed with buffer B and eluted with buffer C. Interaction of GST-SH3 or GST-SH3-Δ1A with the cytoplasmic SH3 domain of ADAM 12 was biotinylated using EZ-Link Sulfo-NHS-biotin (Pierce), according to the manufacturer’s instructions. Briefly, after dialysis against PBS, GST-SH3 was biotinylated with sulpho-NHS-biotin (2 μg/ml, Pierce).

**Immunoprecipitation**—Transfected COS-7 cells were solubilized with buffer A (2 ml of buffer/100-mm plate). Cell extracts were subjected to centrifugation (15,000 × *g*, 20 min), and the supernatant (1 ml) was mixed with protein G-Sepharose (2 μl, Amersham Pharmacia Biotech) and incubated for 1 h at 4 °C (pre-clearing). After removal of protein G-Sepharose, the cell lysate was incubated with anti-ADAM 12 antibody (1.5 μg/ml lysate) or anti-p85α monoclonal antibody (2.5 μg/ml) for 4 h at 4 °C and then with protein G-Sepharose (20 μg/ml) for 30 min at 4 °C. The immunoprecipitates were washed four times with buffer B and eluted with SDS-gel loading buffer. Eluates were analyzed by SDS-PAGE followed by immunoblotting first with anti-p85α, polyclonal antibody or anti-ADAM 12 antibody and then with HRP-coupled secondary antibody.

**Immunoblotting**—Protein samples were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were first washed in blocking buffer (PBS, 3% (w/v) dry milk, and 0.3% (v/v) Tween 20) for 1 h and then incubated with blocking buffer supplemented with 0.5% (v/v) Tween 20 for 1 h. The membranes were washed by a HRP-conjugated secondary antibody. The antigen-antibody complexes were visualized by chemiluminescent detection (SuperSignal West Fico, Pierce). The following concentrations of primary antibodies were used: polyclonal anti-ADAM 12 antibody, 0.3 μg/ml; monoclonal anti-p85α antibody, 0.25 μg/ml; polyclonal anti-p85α antibody, 1 μg/ml.

**Cell Culture and Transfections**—C2C12 and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM glutamine at 37 °C in the presence of 5% CO₂ under humidified atmosphere. Transfection of C2C12 cells (5 × 10⁵ cells/100-mm plate) was performed using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer’s instructions. Expression of the recombinant proteins was analyzed 38 h after transfection.

**Mutagenesis**—Site-directed mutagenesis was performed to introduce alanine substitutions for Pro825, Pro828, Pro833, Pro836, Pro884, or Pro887 of ADAM 12. Mutants were generated by annealing mutagenic primers to a double-stranded plasmid containing ADAM 12-(Δ1–424) insert. Pfx DNA polymerase (Life Technologies, Inc.) was used during PCR to extend the appropriate mutagenic primers. PCR products were digested twice with *DpnI* (Promega, Madison, WI) and then transformed into *E. coli* XL1-Epicurian-Blue Supercompetent cells (Stratagene). Plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). The identity of all of the mutants was verified by DNA sequencing (Iowa State University, Ames, IA).

**Protein Expression and Purification**—All of the GST fusion proteins and CBP-tagged proteins were expressed as soluble forms and purified on glutathione-Sepharose columns (Amersham Pharmacia Biotech) or calmodulin affinity resin (Stratagene), respectively, according to the manufacturers’ instructions.

**Protein Binding under Native Conditions**—Binding of CBP-P1–5 or CBP-P1–4 to GST-SH3 or GST was examined as described earlier (16). To study the interaction of the endogenous p85α with ADAM 12 cytoplasmic tail, C2C12 cells were lysed with buffer A (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM 4-(2-aminoethyl)-benzene-sulfonylfluoride hydrochloride (AEBSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) using 2 ml buffer/100-mm plate. The cell extract was subjected to centrifugation (15,000 × *g*, 20 min), and the supernatant was incubated with glutathione-Sepharose (50 μl/ml lysate) for 1 h. Pre-cleared cell lysate (6 ml) was applied onto column (0.2 ml bed volume) containing GST fusion proteins (0.6 mg). The columns were washed with buffer B (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100) and eluted with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 μl/ml phosphatase and 0.1% Triton X-100 (buffer C). To study the interaction of ADAM 12, ADAM-12(Δ1–424), and ADAM-12(Δ1–424) mutants with the SH3 domain of p85α, pre-cleared lysate from transfected COS-7 (1 ml) was applied onto column (0.1 ml of resin) containing GST-SH3 or GST (0.2 mg). The columns were washed with buffer B and eluted with buffer C.
and is fused to CBP. The SH3 domain of mouse p85α was expressed as a GST fusion protein and purified on glutathione affinity resin. As shown in Fig. 2, the GST-SH3 fusion protein bound directly to CBP-P1–5 but not to a control protein composed of CBP and the cytoplasmic domain of mouse integrin β1A.

Next, we investigated whether the full-length p85α protein and full-length ADAM 12 could participate in the binding. The GST-P1–5 fusion protein was immobilized on a glutathione column, and the lysate from C2C12 mouse myoblasts containing the endogenous p85α was passed through the column. As shown in Fig. 3A, p85α was retained on the GST-P1–5 but not on the GST column, demonstrating that the full-size p85α was capable of interaction with the cytoplasmic domain of mouse integrin β1A.

Moreover, a truncated form ADAM 12, ADAM 12-(D1–424), lacking the N-terminal pro- and metalloprotease domains, containing an exogenous secretion signal, and corresponding to a biologically active form of ADAM 12 described previously (4, 16), bound equally well to GST-SH3 protein (Fig. 3B), suggesting that the pro- and metalloprotease domains are not required for binding. Multiple forms of recombinant ADAM 12 (ranging from ~115 to ~120 kDa; predicted molecular mass, 95 kDa) and ADAM 12-(Δ1–424) (~60–70 kDa; predicted molecular mass, 52 kDa) were the result of the variable extent of protein glycosylation (14, 16).

To gain insight into the localization of p85α-binding sites in ADAM 12, we expressed GST fusion proteins containing the following fragments of the ADAM 12 cytoplasmic domain: P1–4

**Fig. 1. Localization of the SH3 binding motifs in the cytoplasmic domain of ADAM 12.** A, alignment of the amino acid sequences of mouse and human ADAM 12 cytoplasmic domains. The positions of five potential SH3-binding sites are indicated. Sites 2, 4, and 5 contain the consensus sequence of class I ligands for SH3 domains ([R/K]XPPPXXP), and sites 1 and 3 match the consensus sequence of class II SH3 ligands (PX[x]P[X][R/K]). B, schematic representation of the constructs used in this study spanning different fragments of the cytoplasmic domain (CD) of mouse ADAM 12. The positions of the SH3-binding sites (1–5) are indicated by the symbol □.

**Fig. 2. Direct interaction between the cytoplasmic domain of ADAM 12 and the SH3 domain of p85α.** A, Coomassie Blue-stained gel containing purified GST-SH3 fusion protein (lane 1) and GST alone (lane 2). B, binding of the GST-SH3 fusion protein to the cytoplasmic domain of ADAM 12. The P1–5 fragment of ADAM 12 fused to calmodulin-binding peptide (GST-P1–5, lanes 2 and 4) or the cytoplasmic domain of mouse integrin β1A fused to CBP (CBP-β1A, lanes 1 and 3) was immobilized on calmodulin affinity columns. Purified GST-SH3 (lanes 1 and 2) or GST protein (lanes 3 and 4) was loaded on the columns, the columns were washed and eluted with gel loading buffer, and the eluates were subjected to SDS-PAGE and Coomassie Blue staining.
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Interaction between the cytoplasmic domain of ADAM 12 and the full-length p85α (A) and between the SH3 domain of p85α and the full-length ADAM 12 (B). A, the P1–5 fragment of ADAM 12 expressed as a GST fusion protein or GST alone was immobilized on glutathione affinity columns. C2C12 cell lysate (6 ml) was loaded on the columns, the columns were washed and eluted with glutathione-containing buffer (300 μl), and the eluates were subjected to SDS-PAGE and Western blotting with anti-p85α monoclonal antibody. Lane 1 contains 20 μl of the C2C12 cell lysate, and lanes 2 and 3 contain 20 μl of the column eluates. B, left, COS-7 cells were transfected with an expression vector encoding the full-length ADAM 12 (lane 1), ADAM 12 lacking the pro- and metalloprotease domains and containing an exogenous secretion signal (ADAM 12 Δ1–424, lane 2), or with the same vector without insert (Control, lane 3). The lysate from transfected cells was subjected to SDS-PAGE and Western blotting with anti-ADAM 12 antibody. Right, The GST-SH3 fusion protein (lanes 1, 3, and 6) or GST alone (lanes 2, 4, and 6) was immobilized on glutathione affinity columns. The extract from ADAM 12-transfected (lanes 1 and 2), ADAM 12 (Δ1–424)-transfected (lanes 3 and 4), or control cells (lanes 5 and 6) was passed through the columns, the columns were washed and eluted with glutathione-containing buffer, and the eluates were subjected to SDS-PAGE and Western blotting with anti-ADAM 12 antibody.

To further determine which of the SH3 binding motifs was responsible for the interaction with p85α, we used site-directed mutagenesis to disable individual SH3-binding sites in ADAM 12. In each mutant, PXXP motifs, which constitute the core of SH3-binding sites, were replaced with sequences AXXA, leading to a full disruption of any potential interactions involving the mutated sites. Mutants M1, M2, and M5 had a single SH3-binding site disabled (1, 2, or 5, respectively). Double mutants M1M2, M1M5, and M2M5, had only one binding site that remained functional (site 5, 2, or 1, respectively). The triple mutant M1M2M5 had all three SH3-binding sites disabled. The sequences of the regions in ADAM 12 that were subjected to mutagenesis are shown in Fig. 5A.

COS-7 cells were transfected with a vector encoding ADAM 12 (Δ1–424) or the same construct containing M1, M2, M3, M1M2, M1M5, M2M5, or M1M2M5 mutations, as described above. Expression of the recombinant proteins was analyzed by Western blotting using anti-ADAM 12 antibody. As shown in Fig. 5B, the mutations did not affect the stability of the recombinant proteins or the levels of protein expression. As shown further in Fig. 5C, the elimination of a single SH3-binding site in the M1, M2, or M3 mutants did not inhibit binding of ADAM 12 (Δ1–424) to the SH3 domain of p85α. Moreover, simultaneous mutations in any two of the three SH3-binding sites did not abolish the binding. To efficiently inhibit the interaction between ADAM 12 (Δ1–424) and the SH3 domain of p85α, it was necessary to eliminate all three SH3-binding sites.

To examine whether p85α and the cytoplasmic domain of ADAM 12 interact in vivo, C2C12 cells were transfected with a vector encoding ADAM 12 (Δ1–424), ADAM 12 (Δ1–424) triple mutant M1M2M5, or a vector without insert, followed by immunoprecipitation of ADAM 12 (Δ1–424) or p85α and Western blot analysis of the co-immunoprecipitating proteins. The co-immunoprecipitation experiment employed the truncated rather than the full-length version of ADAM 12, because the truncated form, lacking the pro- and metalloprotease domains, is transported to the cell surface much more efficiently than the full-length protein (16, 33). As shown in Fig. 6, p85α was detected in the anti-ADAM 12 immunoprecipitate obtained from ADAM 12 (Δ1–424)-transfected and not from cells transfected with ADAM 12 (Δ1–424) mutant M1M2M5 or from control cells. Reciprocally, ADAM 12 (Δ1–424) was co-immunoprecipitated with anti-p85α antibody, suggesting that the two proteins formed a complex in intact cells.

Finally, we addressed the question of the effect of the interaction with ADAM 12 on PI 3-kinase activity. We reasoned that the interaction of p85α with the cytoplasmic domain of ADAM 12 could recruit PI 3-kinase to the plasma membrane, where the enzyme could get direct access to its lipid substrates. Because the occupancy of the SH3 domain of p85α with proline-rich ligands has not been reported to increase the specific activity of PI 3-kinase (27–29), we decided to measure the activation status of PI 3-kinase by monitoring the amount of PI 3-kinase lipid products in intact cells using a GFP-PH domain.
fusional and L, fusion protein as a probe. The PH domain in the fusion protein was derived from ARNO (Arf nucleotide binding site opener) and was previously shown to bind with a high affinity to PI(3,4,5)P3, one of the major products of PI 3-kinase (30), and was previously shown to bind with a high affinity to p85α, the regulatory subunit of PI 3-kinase, both in vitro and in vivo. We have identified three p85α-binding sites in ADAM 12 involving PXPF motifs located at amino acids 825–828, 833–836, and 884–887. Site-directed mutagenesis established that any one of these sites is sufficient to mediate interaction with p85α in vitro. Moreover, there was very little synergy between the three sites, and the interaction with p85α of ADAM 12 containing three, two, or just one binding site intact was essentially the same. No single site seemed to be critical for the binding, as disruption of any of the three sites did affect the interaction with p85α.

ADAM 12 is the first member of the ADAM family reported to interact with PI 3-kinase. Importantly, several other members of the family contain proline-rich sequences in their cytoplasmic domains and, potentially, they may interact with SH3-containing proteins, including p85α. It has to be stressed, however, that the presence of SH3 binding motifs does not necessarily warrant productive SH3-mediated protein-protein interactions. Specifically, although ADAM 12 contains five legitimate SH3 binding motifs, only three of them (motifs 1, 2, and 5) mediated interactions with p85α, and sites 3 and 4 were nonfunctional. Similarly, we have recently demonstrated that

**Fig. 6. Interaction between ADAM 12 (Δ1-424) and p85α in intact cells.** C2C12 cells were transfected with a vector encoding ADAM 12 (Δ1-424), ADAM 12 (Δ1-424) triple mutant M1M2M5, or with the same vector without insert (Control). A, the lysate from transfected cells was subjected to SDS-PAGE and Western blotting with anti-p85α polyclonal antibody (left) or anti-ADAM 12 antibody (right). B, co-immunoprecipitation of ADAM 12 (Δ1-424) and p85α. The lysate from ADAM 12 (Δ1-424) (lanes 1 and 2), ADAM 12 (Δ1-424) mutant M1M2M5 (lanes 3 and 4), or vector (lanes 5 and 6)-transfected cells was incubated with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) anti-ADAM 12 antibody (top) or anti-p85α monoclonal antibody (bottom) followed by incubation with protein G-Sepharose. The anti-ADAM 12 immunoprecipitates were subjected to Western blotting with anti-p85α polyclonal antibody (top); the anti-p85α immunoprecipitates were analyzed by Western blotting with anti-ADAM 12 antibody (bottom).
the interaction of ADAM 12 with the SH3 domain of protein tyrosine kinase Src required binding sites 1 or 2, whereas sites 3–5 were not active (16).

Activation of PI 3-kinase requires its translocation to the plasma membrane, where the enzyme is positioned in the proximity of its lipid substrates. The most common mechanism of the translocation to the membrane involves the interaction of SH2 domains in the regulatory subunit of PI 3-kinase with activated, tyrosine-phosphorylated growth factor receptors or adaptor molecules (27–29). In addition to mediating recruitment to the membrane, interaction of SH2 domains with phosphopeptides further increases the specific activity of the catalytic subunit of PI 3-kinase, leading to full activation of the enzyme (35, 36). It has to be stressed, however, that translocation to the plasma membrane alone is sufficient to activate PI 3-kinase, as demonstrated by targeting of the p110 catalytic subunit to the membrane by either N-terminal myristoylation or C-terminal farnesylation (37). These membrane-bound forms of p110 produced constitutively active PI 3-kinases and induced PI 3-kinase-dependent responses in the absence of growth factor stimulation. Transmembrane ADAM 12, by providing docking sites for the SH3 domain of p85α, could therefore play an important role in the activation of PI 3-kinase by directly recruiting it to the membrane. At the present moment, it is not clear whether other mechanisms contribute further to the activation of PI 3-kinase at the membrane. Nevertheless, because PI 3-kinase is critical for terminal differentiation of myoblasts (17–19), and because expression of ADAM 12 is dramatically up-regulated at the onset of myoblast differentiation (4, 6, 8), ADAM 12-mediated recruitment to the mem-

**FIG. 7.** Activation of PI 3-kinase by ADAM 12. C2C12 cells were co-transfected with expression vectors encoding ADAM 12-(Δ1–424) and the pleckstrin homology domain of ARNO fused to GFP (A–D, K, and L), the myristoylated cytoplasmic domain of ADAM 12, and GFP-PH (E and F), GFP-PH alone (G and H), or ADAM 12-(Δ1–424) triple mutant M1M2M5 and GFP-PH (I and J). In K and L, cells were incubated with the PI 3-kinase inhibitor LY294002. Transfected cells were stained with anti-ADAM 12 rabbit antibody and rhodamine-conjugated anti-rabbit IgG antibody (A, C, E, G, I, and K). GFP-PH was visualized by direct fluorescence microscopy (B, D, F, H, J, and L). Note the presence of GFP-PH at the plasma membrane in ADAM 12-(Δ1–424)- or myristoylated cytoplasmic domain-transfected cells (arrows in B, D, and F) and the lack of such localization in cells transfected with GFP-PH only (H) or in cells transfected with mutant ADAM 12-(Δ1–424) or incubated with LY294002 (arrowheads in J and L). M, Western blot analysis of the myristoylated cytoplasmic domain of ADAM 12 (myr-CD) and GFP-PH in C2C12 cells.
brane may constitute one of the regulatory mechanisms for PI 3-kinase during the differentiation process.

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