The Tyrosine Kinase Substrate eps15 Is Constitutively Associated with the Plasma Membrane Adaptor AP-2

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Abstract. The ubiquitous eps15 protein was initially described as a substrate of the EGF receptor kinase. Its functions are not yet delineated and this work provides evidence for its possible role in endocytosis. A novel anti-eps15 antibody, 6G4, coimmunoprecipitated proteins of molecular mass 102 kD. In human cells, these proteins were identified as the α- and β-adaptins of the AP-2 complex on the basis of their NH2-terminal sequence and their immunoreactivity with anti-α- and anti-β-adaptin antibodies but not with anti-γ-adaptin antibody. In addition, the anti-eps15 antibody coimmunoprecipitated metabolically labeled polypeptides with molecular mass of 50 and 17 kD, comparable to those of the two other components of the AP-2 complex, μ2 and δ2. Constitutive association of eps15 with AP-2 was confirmed by two sets of experiments. First, eps15 was detected in immunoprecipitates of anti-α- and anti-β-adaptin antibodies. Second, α- and β- but not γ-adaptins were precipitated by a glutathione-S-transferase eps15 fusion protein. The association of eps15 with AP-2 was ubiquitous and conserved between species, since it was observed in human lymphocytes and epithelial cells and in murine NIH3T3 fibroblasts. Our results are in keeping with a recent study showing homology between the NH2-terminal domains of eps15 and the product of the gene END3, involved in clathrin-mediated endocytosis of the pheromone α factor in Saccharomyces cerevisiae, and suggest a possible role for eps15 in clathrin-mediated endocytosis in mammals.

The eps15 protein was first identified by an expression cloning approach as a tyrosine-phosphorylated substrate of the EGF receptor (EGFR) (Fazioli et al., 1993). Cloning of the human eps15-coding sequence subsequently showed a close structural similarity, with 90% identity of nucleotide and predicted amino acid sequences with the mouse protein (Wong et al., 1994). Using the human coding sequence as a probe, Wong et al. (1994) demonstrated that eps15 is a member of a gene family that is highly conserved during evolution, as sequences are detected in unicellular organisms such as Saccharomyces cerevisiae (Wong et al., 1994). The structural analysis of eps15 indicates that it is a cytosolic protein made of three distinct domains: an NH2-terminal domain composed of three imperfect repeats, including a candidate tyrosine phosphorylation site and two calcium-binding domains of the EF-hand type; a central domain characterized by the presence of heptads required for coiled-coil structure; and a COOH-terminal domain in which the aspartate-proline-phenylalanine (DPF) sequence is repeated 13–15 times, a motif observed in several methyl-transferases (Fazioli et al., 1993; Wong et al., 1994). This structure does not provide immediate clues as to the physiological function(s) of eps15. However, there is evidence for its importance in signal transduction, since eps15 is tyrosine phosphorylated in response to stimulation of the EGF- and PDGF-receptors (Fazioli et al., 1993). It may also have a role in cell proliferation since overexpression of eps15 in NIH3T3 fibroblasts caused transformation albeit with low efficiency (Fazioli et al., 1993); and the human gene for eps15, which maps to chromosome 1p32 (Wong et al., 1994), is rearranged with the HRX/ALL1/MLL gene in the t(1;11)(p32,q23) translocation found in acute myeloid leukemias (Bernard et al., 1994).

The present report provides evidence for a possible function of eps15 in endocytosis, as it shows that eps15 is constitutively associated with an ubiquitous protein complex, AP-2, which participates in clathrin-dependent endocytosis (Keen, 1990; Robinson, 1992). Thus, a new anti-eps15 mAb, 6G4, coimmunoprecipitated in several cell types the eps15 protein with polypeptides of 102 kD. On
the basis of their NH2-terminal sequence and their immunoactivity, these polypeptides were identified as the α- and β-adaptins of the AP-2 complex. The interaction between eps15 and AP-2 was confirmed using either anti-adaptin antibodies to communoprecipitate eps15 or a glutathione-S-transferase-eps15 fusion protein (GST-eps15) to precipitate the adaptins of AP-2.

Materials and Methods

Cells

Stimulation and long-term culture of normal human T cells were performed as described (Cerf-Bensussan et al., 1992). Sublines of the human leukemic T cell line MOLT16 (gift of Dr. Minowada, Fujisaki Cell Center, Japan), a human B cell line transformed by the Epstein Barr Virus (EBV) (gift of Dr. F. Le Deist, Hôpital Necker-Enfants, Paris, France), the DLD1 human epithelial cell line (gift of Dr. L. L. Mayer, Mount Sinai Medical Center, NY), and the murine NIH3T3 cell line were grown in RPMI 1640 or in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD).

Antibodies

The new mAb 6G4 (IgG2a) was obtained by fusion of SP2/0 myeloma cells with spleen cells of Balb/c mice immunized with a subline of MOLT16 cells. This antibody reacted with an ubiquitous 140-kD protein and identified a full-length cDNA clone encoding a 140-kD protein identical to eps15 (manuscript in preparation). This antibody reacts with a glutathione-S-transferase fusion protein containing the entire sequence of eps15, as shown in Fig. 7. The anti-eps15 mAb, obtained by immunization against a 19.8-kD protein fragment corresponding to residues 717-896 of murine eps15, was from Affinity Research Products Ltd. (Nottingham, UK).

The mouse anti-α-adaptins mAbs, AC1-M11, pan-anti-α-adaptin, AC2-M15, anti-α-adaptin A and the affinity-purified rabbit anti-α-adaptin C immune serum (Robinson, 1987; Sorkin et al., 1993, 1995) were kind gifts from Dr. M. S. Robinson. Anti-β-adaptin mAb, 1001 (Ahle et al., 1988), was first provided by Dr. E. Ungewickell and subsequently obtained, as anti-β-adaptin (clone 1002) and anti-γ-adaptin (clone 1003) mAbs, from Sigma (Saint Quentin Fallavier, France). Rabbit anti-α-C- and anti-β-adaptins Abs13 and Ab15 (Sorkin et al., 1995) were given by Dr. A. Sorkin. The pan-anti-COP mAbs (M3A5 and mAD) (Allan and Kreis, 1986; Pepperkok et al., 1993) were given by Dr. T. Kreis.

Construction of the eps15 Fusion Protein

The human cDNA of eps15 subcloned in pBluescript II KS (PBS-eps15) was obtained in the laboratory (manuscript in preparation). Fusion proteins were derived from eps15 using the glutathione-S-transferase (GST) gene translation system and the PGEX.1 vector (Pharmacia-LKB, Les Ulis, France). A DNA fragment encoding the first 96 NH2-terminal amino acids of eps15 was generated by PCR using PBS-eps15 as a template. A BamHI site was introduced in the upper primer (nucleotides 8-33 of the published sequence) and a XhoI site in the lower primer (nucleotides 300-319). The PCR product was purified, digested, and subcloned into the BamHI/XhoI sites of the PGEX.1 vector. The construct was checked by nucleotide sequencing (Circumvent, Biolabs). The open-reading frame of eps15 contains a unique EcoRI site located in position 225. Another EcoRI site is located just after the stop codon (nucleotides 2714-2720). The full-length GST-eps15 construct was therefore obtained by introducing the main 2.6-kb EcoRI fragment of the open-reading frame of eps15 cDNA into the EcoRI site of the NH2 domain subcloned in PGEX.5.1 (Fig. 7 A).

Induction and Production of the Fusion Proteins

DH5α bacteria transformed with the PGEX.5.1 construct were induced to produce the fusion proteins. A single colony was grown overnight in LB containing 100 μg/ml of ampicillin (Boehringer-Mannheim, Meylan, France). The bacteria were then diluted 1:10 in the same medium and grown to an OD600 of 0.4. After induction of the fusion proteins with 0.1 mM of IPTG (Bioprobe Systems, Nontreuil, France) for 3-4 h at 37°C, the bacteria were centrifuged at 1,600 g at 4°C for 10 min. The pellet was re-suspended in cold PBS containing 1 mM PMSF and then mildly sonicated. Triton X-100 (Sigma) was added at a final concentration of 1% and the sonicate was centrifuged for 5 minutes at 10,000 g. The supernatant was tested by immunoblotting and used to purify the GST fusion proteins on glutathione Sepharose 4B using the Bulk GST Purification Module (Pharmacia-LKB, Les Ulis, France) according to the manufacturer's instructions. The GST-eps15 protein had the expected size (170 kD) and reacted with the 6G4 antibody and with the commercial mAb produced against the COOH-terminal peptide of eps15 (Fig. 7 B).

Biochemical Procedures

Procedures for biosynthetic labeling with 35S-labeled amino acids (Trans-label, Dositék-ICN, Les Ulis, France), membrane sulfobutilization in 0.5% Triton X-100 or NP-40-containing buffers (Sigma), immunoprecipitation with mAbs coupled to protein A-Sepharose or to CNBr-activated Sepharose beads (Pharmacia-LKB), and SDS-PAGE analysis have been described elsewhere (Cerf-Bensussan et al., 1986, 1987, 1992). For precipitation with fusion proteins, cell lysates were first cleared with GST (4 μg/20 × 106 cells) and glutathione–Sepharose 4B beads (20 μl/20 × 106 cells) for 8 h and then precipitated with GST-eps15 (4 μg/20 × 106 cells) and glutathione–Sepharose 4B beads (20 μl/20 × 106 cells) overnight. Precipitates were washed four times in 50 mM Tris, pH 8, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF and twice in 40 mM Heps, pH 8, and analyzed by Western blotting.

Two-dimensional gel electrophoresis of biosynthetically labeled proteins was performed using a BioRad Miniprotein™ II 2D apparatus according to the manufacturer's instructions. Briefly, the samples were resolved by nonequilibrium pH gel electrophoresis (NEPHGE) (20% vol/vol, pH 3-10, and 80% vol/vol, pH 5-7, ampholines) in nonreducing conditions, followed by SDS-PAGE in 6.5% polyacrylamide gel in nonreducing conditions.

For Western blotting, acrylamide gels were transferred onto nitrocellulose membranes (Schleicher-Schuell, Eschweil, France) in 10 mM Tris, 0.2 M glycine, and 30% methanol for 3 h. Non-specific binding sites were blocked by incubation in Tris- HCL pH 7.6, containing 5% BSA and 0.2% Tween (Sigma). The blots were then sequentially incubated for one hour, either with mouse mAbs at the indicated dilutions, followed by peroxidase-labeled sheep anti–mouse immunoglobulin antiserum (1:20,000) (Amersham Buchler GmbH, Braunschweig, Germany), or with rabbit anti-sera at the indicated dilutions, followed by peroxidase-labeled swine anti-rabbit immunoglobulin antiserum (1:5,000) (Dakopatts, Trappes, France). Labeled bands were revealed using ECL (Amersham).

Purification of eps15-associated proteins was performed according to a modification of a published procedure (Cerf-Bensussan et al., 1992). Briefly, NP-40 lysates of 20 × 106 MOLT16 cells were incubated with 6G4 antibody coupled to CNBr-activated beads (Pharmacia) (5 mg mAb/ml of beads). After extensive washing of the beads, the proteins were eluted in 100 mM glycine, pH 2.1, 1% NP-40, and precipitated with 15% TCA. The protein pellet was washed in acetone, resuspended in electrophoresis sample buffer containing 50 mM DTT, and then subjected to a 6.5% SDS-PAGE. The proteins were then transferred to a PVDF membrane (Pro-blott, Applied Biosystems, Inc., Foster City, CA) in 10% CAPS buffer (3-cyclohexylamino-1-propanesulfonic acid; Sigma) containing 10% methanol. Transferred proteins were revealed with Coomassie blue; the bands to be analyzed were excised and directly processed for amino acid sequence determination in a sequencer (model 477A; Applied Biosystems) equipped with an on-line phenylthiohydantoin analyzer (model 1201; Applied Biosystems). Comparison of the peptide sequences with a protein data bank was performed using the Biscane program provided by CITII (Paris).

Results

The Anti-eps15 Monoclonal Antibody 6G4 Coimmunoprecipitates a 102-kD Polypeptide

In metabolically labeled activated human T cells, the antibody 6G4 immunoprecipitated the 140-kD eps15 protein and a polypeptide that migrated at 102 kD under reducing conditions (Fig. 1 A, lane 2). These proteins comigrated at 125 kD under nonreducing conditions (Fig. 1 A, lane 4), raising the possibility that the 102-kD polypeptide ob-
Figure 1. Association of a 102 kD polypeptide with the 140 kD eps15 protein. (A) Human activated T cells were biosynthetically labeled for 45 min with 35S-labeled amino acids. After a 5-h chase, cells were lysed in 50 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, containing a mix of protease inhibitors (10 μg/ml leupeptin, aprotinin, pepstatin, 50 μg/ml trypsin inhibitor, 4 mM PMSF). The cell lysate was cleared with protein A-Sepharose (control, lanes 1 and 3), and immunoprecipitated with the anti-eps15 antibody, 6G4, coupled to protein A-Sepharose (lanes 2 and 4). Precipitates were run on a 6% SDS-PAGE under reducing (R; lanes 1 and 2) or nonreducing (NR; lanes 3 and 4) conditions. (B) A subline of MOLT16 cells was biosynthetically labeled for 2 h and chased for 30 min. Cell lysates were immunoprecipitated with 6G4 coupled to protein A-Sepharose and separated by nonequilibrium pH gel electrophoresis (NEPHGE) in the first dimension and by SDS-PAGE in a 6.5% gel in the second dimension (2D). Both migrations were run under nonreducing conditions (NR). This allowed for the separation of the 125-kD band immunoprecipitated by 6G4 in nonreducing conditions into a major spot A, and a minor spot B, with a distinct pI.

The 102-kD Band Immunoprecipitated by the Anti-eps15 Antibody 6G4 Contains the α-Adaptins

Using the 6G4 antibody, it was possible to coimmunoprecipitate sufficient amounts of the 102-kD band from cell lysates of the leukemic T cell line MOLT16 to allow NH2 terminus microsequencing. Comparison of the sequences obtained in three runs of microsequencing with a protein data bank using the Bisance program revealed a strong homology with the NH2 terminus of mouse and rat α-adaptins (Kirchhausen et al., 1989; Robinson, 1989; Tucker et al., 1990) (Fig. 2 A). Only two amino acids were clearly different from the mouse sequence: the proline following the initial methionine and the serine in the fifth position. The most likely amino acids in these positions were a serine and a glutamic acid respectively. α-adaptins are intracellular proteins highly preserved among species. The presence of α-adaptins in the 102-kD band immunoprecipitated by 6G4 could therefore be confirmed by immunoblotting using AC1-M11, a mAb that recognizes mouse α-adaptins and cross-reacts with their human counterparts (Robinson, 1987) (Fig. 2 B, lane 3). In mice, two isoforms of α-adaptins, A and C, have been described. The α-adaptin C is ubiquitous. The α-adaptin A has two alternatively spliced variants, a long one restricted to the brain, and a shorter ubiquitous one that runs on gels with the same mobility as the α-adaptin C (Robinson, 1989, and personal communication). This prompted us to compare the reactivity of the 102-kD band with three anti-α-adaptin antibodies: the AC1-M11 mAb that recognizes the two isoforms A and C of mouse and human α-adaptins (Robinson, 1989); AC2-M15, a mAb specific for α-adaptin-A (Sorkin et al., 1993); and C8, a polyclonal antibody specific for the C isoform (M. Robinson, personal communication). Immunoblotting experiments indicated that the 102 kD band immunoprecipitated from MOLT16 cells reacted with all three antibodies (Fig. 2, B and C).

The Anti-eps15 Antibody 6G4 Coimmunoprecipitates the Other Components of AP-2

α-adaptins are components of AP-2, a heterotetrameric complex that comprises another protein with a mobility comparable to that of α-adaptins, the β-adaptin (Kirchhausen et al., 1989; Ponnambalan et al., 1990), and two smaller polypeptides with a molecular mass of 50 (μ2) (Thurieau et al., 1988) and 17 kD (tr2) (Kirchhausen et al., 1991). Because proteins composing AP-2 dissociate only under strong denaturing conditions, one could expect to find the other components of the AP-2 complex coimmunoprecipitated by the 6G4 antibody. In biosynthetic labeling experiments using a 60-min pulse followed by a 30-min chase, additional bands with a molecular mass of 50 and 17 kD comparable to those of the μ2 and tr2 chains of AP-2 were coimmunoprecipitated by 6G4 (Fig. 3 A, lane 2). Furthermore, in immunoblotting, the 102-kD band immunoprecipitated by 6G4 from the T cell line MOLT16 reacted with the anti-β-adaptin mAb, 100/1 (Fig. 3 B, lane 3). The latter antibody recognizes the β2 adaptin of the AP-2 plasma membrane complex and a highly homologous protein, the β1 adaptin, which belongs to a distinct heterotetrameric complex, AP-1, present in clathrin-coated vesicles of the trans-Golgi network (Ahle et al., 1988; Kirchhausen et al., 1989; Ponnambalan et al., 1990). In the latter complex, the β1 adaptin is associated with three polypeptides distinct from the other three AP-2 components. Particularly, α-adaptin is replaced in AP-1 by γ-adaptin (Robinson, 1990). The 102-kD band immunoprecipitated from the T cell line MOLT16 did not react with an antibody directed against γ-adaptin (Fig. 3 B, lane 4). It did not react either with M3A5 or mAD (Fig. 2 C and not shown), two antibodies specific for β-COP, a protein of the coatamer of non-clathrin-coated vesicles that shows some homology...
Identification of the eps15-associated 102-kD polypeptide with the α-adaptin. (A) The 102-kD band was immunopurified using 6G4 antibody as described in Materials and Methods. The NH$_2$-terminal sequences of the 102-kD band provided by three distinct runs of microsequencing were compared with the NH$_2$ terminus common to the two isoforms, A and C, of mouse α-adaptins. (B and C) Cold lysates of 120 × 10$^6$ (B) or 100 × 10$^6$ MOLT16 cells (C) were immunoprecipitated with 6G4 coupled to CNBrSe. Samples were first separated by SDS-PAGE under reducing conditions in a 6.5% gel and then transferred to nitrocellulose. In B, blots were hybridized with an irrelevant mouse monoclonal antibody (lane 1, control mouse mAb), with the anti-eps15 antibody 6G4 (0.5 μg/ml) (lane 2), with the pan-anti-α-adaptin antibody AC1-M11 (ascites, 1:2,000) (lane 3), followed by peroxidase-labeled sheep anti-mouse immunoglobulin antiserum (1:20,000). In C, the blots were hybridized with the pan-anti-α-adaptin antibody AC1-M11 (lane 1), with the AC2-M15 antibody specific for α-adaptin A (ascites, 1:50) (lane 2), with an irrelevant mouse monoclonal antibody (lane 3), then with peroxidase-labeled sheep anti-mouse immunoglobulin antiserum (1:20,000). In lane 4, the blot was sequentially incubated with C8, an affinity-purified rabbit antiserum specific for α-adaptin C (1:250) and with a swine anti-rabbit immunoglobulin antiserum coupled to peroxidase (1:5,000). A control blot incubated with the latter antiserum only is shown in lane 5. Labeled bands were revealed using ECL.

3), followed by peroxidase-labeled sheep anti–mouse immunoglobulin antiserum (1:20,000). In C, the blots were hybridized with the pan-anti-α-adaptin antibody AC1-M11 (lane 1), with the AC2-M15 antibody specific for α-adaptin A (ascites, 1:50) (lane 2), with an irrelevant mouse monoclonal antibody (lane 3), then with peroxidase-labeled sheep anti–mouse immunoglobulin antiserum (1:20,000). In lane 4, the blot was sequentially incubated with C8, an affinity-purified rabbit antiserum specific for α-adaptin C (1:250) and with a swine anti–rabbit immunoglobulin antiserum coupled to peroxidase (1:5,000). A control blot incubated with the latter antiserum only is shown in lane 5. Labeled bands were revealed using ECL.

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Figure 4. Association of eps15 and adaptins of the AP-2 complex in human B lymphocyte and epithelial cell lines. EBV-infected B cells (40 × 10^6) and DLD1 epithelial cells (20 × 10^6) were biosynthetically labeled for 45 min with 35S-labeled amino acids. After a 3-h chase, cells were lysed in 0.5% NP-40, containing a mixture of protease inhibitors. Cell lysates were cleared with nonimmune mouse immunoglobulins coupled to CNBr-activated Sepharose beads (Ig-CNBr, lane 1) and immunoprecipitated with the anti-eps15 antibody, 6G4, coupled to CNBr-activated Sepharose beads (6G4-CNBr, lane 2). Alternatively, cold lysates of EBV-infected B cells (100 × 10^6) and of DLD1 cells (40 × 10^6) were immunoprecipitated with 6G4 coupled to CNBr-activated Sepharose beads, separated on 6.5% SDS-PAGE under reducing conditions, transferred onto nitrocellulose, and immunoblotted with 6G4 (lane 3), or with mAbs against α-adaptin, (mAb ACM1-M11, lane 4), β-adaptin (mAb 100/1, lane 5) and γ-adaptin (mAb 100/3, lane 6).

man DLD1 epithelial cells (Fig. 4 B), 6G4 immunoprecipitated two bands of molecular mass 140 and 102 kD under reducing conditions (lane 2). The 140-kD band reacted with 6G4 (lane 3) and the 102-kD band with anti-α-adaptin AC1-M11 mAb (lane 4) and anti-β-adaptin 100/1 mAb (lane 5), but not with anti-γ-adaptin 100/3 mAb (lane 6), indicating that the association of eps15 with AP-2 was not restricted to T cells.

The 140-kD eps15 Protein Is Coimmunoprecipitated by Anti-α- and Anti-β-adaptin Antibodies

To confirm the association of eps15 with the AP-2 complex, MOLT16 cells were immunoprecipitated with the Ab32 anti-β-adaptin rabbit anti- serum. Previous studies have shown that this antibody immunoprecipitates very efficiently murine as well as human β-adaptins (Sorkin et al., 1995, and personal communication). As shown in Fig. 5, this antibody immunoprecipitated adaptins from MOLT16 cells and coimmunoprecipitated a small amount of a 140-kD polypeptide immunoreactive with 6G4 (lane 7 and 8). Sequential immunoprecipitation using 6G4 and the Ab32 antibody indicated that only a small amount of eps15 was associated with α-adaptins in MOLT16 cells. Indeed, when the cell lysate was first depleted of adaptins with Ab32, a large amount of eps15 could still be immunoprecipitated with 6G4 (Fig. 5, lane 5). Conversely, when the cell lysate was first depleted of eps15 with 6G4, significant amounts of α-adaptins were immunoprecipitated by Ab32 (Fig. 5, lane 6).

In a previous study, an anti-αC adaptin rabbit antise-
Immunoprecipitation of eps15 by anti-α-adaptin antibody Ab31 in murine NIH3T3 fibroblasts. Two million NIH3T3 cell lysates were treated with protein A-Sepharose (lane 1) and immunoprecipitated with 5 μg of 6G4 coupled to protein A-Sepharose or treated with nonimmune rabbit immunoglobulins coupled to protein A-Sepharose (lane 4) and immunoprecipitated with 10 μl of rabbit anti-mouse αC-adaptin immune serum Ab31 (lane 3) coupled to protein A-Sepharose. Precipitates were separated on 6.5% SDS-PAGE under reducing conditions and transferred onto nitrocellulose. The upper part of the membrane was immunoblotted with 6G4 and the lower part with the anti-α-adaptin mAb 100/2.

Figure 6. Immunoprecipitation of eps15 by anti-α-adaptin antibody Ab31 in murine NIH3T3 fibroblasts. Two million NIH3T3 cell lysates were treated with protein A-Sepharose (lane 1) and immunoprecipitated with 5 μg of 6G4 coupled to protein A-Sepharose or treated with nonimmune rabbit immunoglobulins coupled to protein A-Sepharose (lane 4) and immunoprecipitated with 10 μl of rabbit anti-mouse αC-adaptin immune serum Ab31 (lane 3) coupled to protein A-Sepharose. Precipitates were separated on 6.5% SDS-PAGE under reducing conditions and transferred onto nitrocellulose. The upper part of the membrane was immunoblotted with 6G4 and the lower part with the anti-α-adaptin mAb 100/2.

A GST-eps15 Fusion Protein Precipitates the Adaptins of the AP-2 Complex

A GST fusion protein encoding the full length of the eps15 protein was obtained as described in Materials and Methods and in Fig. 7 A. The GST-eps15 protein had the expected size of 170 kD. It reacted with the 6G4 antibody that recognizes an epitope located in the first 97 amino acids of eps15 (data not shown) and with the commercial mAb produced against the COOH-terminal peptide of eps15 (Fig. 7 B). This protein was used to precipitate MOLT16 cell lysates. Immunoblotting with anti-adaptin antibodies showed that α-, β-, but not γ-adaptins were precipitated by the GST-eps15 fusion protein. No adaptins were detected in the control precipitates with GST protein alone. These results indicated that, in the absence of antibodies, eps15 can associate with the adaptins of the AP-2 complex.

Discussion

Our results indicate that the tyrosine kinase substrate eps15 (Fazioli et al., 1993; Wong et al., 1994) can associate, in lymphocytes, in epithelial cells and in fibroblasts, with consistent with previous observations in mice using other anti-eps15 antibodies (Fazioli et al., 1993). In addition, 6G4 coimmunoprecipitated a band of ~102 kD immunoreactive with the anti-α-adaptin mAb 100/2. Since 6G4 reacted with mouse eps15, we examined whether the 145-kD protein observed after immunoprecipitation of biosynthetically labeled NIH3T3 cells with anti-αC adaptin rabbit anti-serum was recognized by 6G4. As shown in Fig. 6 (lane 3), this serum immunoprecipitated α-adaptins from NIH3T3, together with a major 145-kD band and a minor 120-kD band, which both reacted with 6G4.

Discussion

Our results indicate that the tyrosine kinase substrate eps15 (Fazioli et al., 1993; Wong et al., 1994) can associate, in lymphocytes, in epithelial cells and in fibroblasts, with
the AP-2 complex involved in clathrin-mediated endocytosis (Keen, 1990; Robinson, 1992). Thus, a new anti-eps15 antibody coimmunoprecipitated the 140-kD eps15 protein with 102-kD polypeptides from human lymphocytes and epithelial cells (Keen, 1990; Robinson, 1992). Out of 18 amino acids identified in the NH2 terminus of the human 102-kD band, 14 were identical to the corresponding amino acids in the NH2 terminus of murine α-adaptins (Robinson, 1989). Immunoblotting using anti-murine α-adaptin antibodies, which cross-react between species, confirmed that the 102-kD band from human MOLT16 T lymphocytes contained the α-adaptins and revealed the presence of the two isoforms with an ubiquitous distribution, the short isoform A and the C isoform. The presence of α-adaptins was also detected in the 102-kD band coimmunoprecipitated by the anti-eps15 antibody, 6G4, from human EBV-infected B cells and epithelial cells. Immunoblotting indicated that the 102-kD band contains not only α-adaptins but also β-adaptin, the other major component of AP-2, which dissociates from the α-adaptins only under strong denaturing conditions (Kirchhausen et al., 1989). This result explains the heterogeneous appearance of the 102 kD band in one-dimension SDS-PAGE. The lack of resolution of the three adaptins by isoelectrofocusing is consistent with previous experiments (M. Robinson, personal communication). Blockage of the NH2 terminus of the β-adaptin has previously been observed (Kirchhausen et al., 1989) and likely explains why the corresponding microsequence was not detected in the 102-kD band. As indicated above, the antibody used to detect β-adaptin reacts not only with the β2 adaptin of the plasma membrane AP-2 complex, but also with the β1 adaptin, a highly homologous protein (Kirchhausen et al., 1989; Ponnambalan et al., 1990) that associates with the γ-adaptin (Robinson, 1990) to form the AP-1 complex. In contrast with AP-2 associated with clathrin-coated endocytic vesicles derived from the plasma membrane, AP-1 is associated with clathrin-coated vesicles that bud from the trans-Golgi (Ahle et al., 1988; Robinson, 1992). The absence of detectable γ-adaptin in the 102-kD band indicates that the proteins associated with eps15 belong to the AP-2 plasma membrane complex but not to the AP-1 Golgi adaptor complex. The immunoprecipitation of proteins with a molecular mass of 50 and 17 kD in biosynthetic labeling studies suggested that the μ2 and σ2 components of the AP-2 complex may also be coimmunoprecipitated by the anti-eps15 antibody, 6G4.

Association of eps15 with adaptins was confirmed by two sets of experiments. First, anti-adaptin antibodies coimmunoprecipitated the eps15 protein. Coimmunoprecipitation was observed in human cells using the anti-mouse and human β-adaptin antibody, Ab32, as well as in mouse fibroblasts using the anti-mouse αC-adaptin antibody, Ab31. Second, association of eps15 with AP-2 was directly demonstrated by showing precipitation of α- and β- but not γ-adaptins by a GST-eps15 fusion protein. These results indicate that immunoprecipitation of adaptins by 6G4 is not due to a cross-reactivity of this antibody with proteins of the AP-2 complex and can be observed without the use of antibodies. Furthermore, they indicate that the association of eps15 and AP-2 is not restricted to human cells, but is conserved between species.

The function(s) of eps15 are not yet known. On the one hand, its phosphorylation in response to EGF or PDGF suggests a possible function in signal transduction via these receptors (Fazioli et al., 1993). The recent demonstration that eps15 can bind the SH3 domain of the protooncogene Crk, an adaptor protein containing two SH3 domains and an SH2 domain, sustains this hypothesis (Schumacher et al., 1995). On the other hand, eps15 may play a role in endocytosis. Thus, there is significant similarity (62% similarity, 41% identity) between the three imperfectly repeated regions of 95–97 amino acids constituting the NH2-terminal domain of eps15 and the entire NH2-terminal domain of End3p, a protein required for internalization of α-factor via a clathrin-mediated pathway in Saccharomyces cerevisiae (Tan et al., 1993; Bénédetti et al., 1994). Our results showing association of eps15 with AP-2, a protein complex required for recruitment of receptors into clathrin-coated pits, suggest a function for this protein in clathrin-mediated endocytosis in mammals. The mechanisms by which AP-2 interacts with receptors have not been entirely elucidated. On the one hand, AP-2 binds clathrin heavy chains via β-adaptin and promotes association of clathrin triskelions that coat endocytic vesicles (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993). On the other hand, AP-2 could bind the intracytoplasmic tail of internalized receptors and thereby directly promote their recruitment into clathrin-coated pits. In agreement with this hypothesis, direct albeit weak interactions between AP-2 and some internalized receptors have been observed in vitro using purified AP-2 and receptors (Pearse, 1988; Glickman et al., 1989; Beltzer and Spiess, 1991; Sosa et al., 1993; Nesterov et al., 1995). More detailed studies have been performed with the EGFR. Coimmunoprecipitation of EGFR with AP-2 in cell lysates was observed after stimulation by EGF at 37°C, a condition known to induce EGFR endocytosis (Sorkin and Carpenter, 1993; Sorkin et al., 1995). Yet, in a cell-free assay, recruitment of EGFR into coated pits required not only the tyrosine kinase activity of the receptor but also an as yet unidentified cytosolic tyrosine kinase substrate of the EGFR (Lamaze and Schmid, 1995). Unidentified cytosolic components were also required for recruitment of the constitutively endocytosed transferrin receptor into coated pits (Lamaze et al., 1993). These observations and the difficulties encountered in demonstrating in vivo association of AP-2 with endocytosed membrane receptors other than EGFR suggest that additional molecules able to mediate interactions with AP-2 are needed in vivo to recruit endocytosed receptors into coated pits. Eps15 appears to be one possible candidate for this function. Two observations indirectly support a role for eps15 in the recruitment of EGFR by AP-2. First, Fazioli et al. observed efficient tyrosine-phosphorylation of eps15 by the EGFR, which is rapidly endocytosed but not by an EGFR/erbB-2 chimera, which has a much lower rate of endocytosis (Fazioli et al., 1993; Sorkin et al., 1993). Second, in vitro studies showed association between activated EGFR-kinase and eps15 via the protooncogene Crk (Schumacher et al., 1995). However, it is noteworthy that tyrosine-phosphorylation of eps15 is not required for binding of AP-2. AP-2-eps15 complexes were detected in unstimulated MOLT16 T cells where eps15 is not tyrosine-phosphorylated (data not
shown). Furthermore, the unphosphorylated GST-eps15 fusion protein produced by bacteria was able to precipitate the adaptins of AP-2. Tyrosine-phosphorylation and/or other modifications of eps15 may perhaps be needed to induce the recruitment of the complexes or of AP-2 by endocytosed receptors. Functional studies currently in progress will help to directly assess the role of eps15 in endocytosis.

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