Human Nischarin/Imidazoline Receptor Antisera-selected Protein Is Targeted to the Endosomes by a Combined Action of a PX Domain and a Coiled-coil Region*

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Around 50 mammalian and 15 yeast proteins are known to contain the phox (PX) domain, the majority (about 30) of which is classified as sorting nexins (SNXs). The PX domain, a hallmark of these proteins, is a conserved stretch of about 120 amino acids and is recently shown to mediate phosphoinositide binding. A few PX domain proteins (including some SNXs) have been shown to participate in diverse cellular processes such as protein sorting, signal transduction, and vesicle fusion. In this report, we present our results supporting a role of human IRAS to act as a SNX. The mouse homologue, previously identified as Nischarin, has been shown to interact with the α5 subunit of integrin and inhibit cell migration (Alahari, S. K., Lee J. W., and Juliano R. L. (2000) J. Cell Biol. 51, 1141–1154). Its human homologue (imidazoline receptor antisera-selected (IRAS)), on the other hand, contains an NH2-terminal extension and is a larger protein of 1504 amino acids consisting of an NH2-terminal PX domain, 5 putative leucine-rich repeats, a predicted coiled-coil domain, and a long COOH-terminal region. We show that it has the ability to homo-oligomerize via its coiled-coil region. The PX domain of IRAS is essential for association with phosphatidylinositol 3-phosphate-enriched endosomal membranes. However, the PX domain of IRAS alone is insufficient for its localization to endosomes, unless the coiled-coil domain was included or it is artificially dimerized by glutathione S-transferase. Interaction of human IRAS with α5 integrin is not affected by the NH2-terminal extension, and overexpression of IRAS could cause a redistribution of surface α5 integrin to intracellular endosomal structures.

Regulation of vesicular trafficking, cell proliferation, receptor signaling, and other cellular functions may be exerted by controlling the recruitment of cytosolic proteins to their respective intracellular membrane compartments, via various protein domains capable of interaction with phosphorylated derivatives of phosphoinositides (PtdIns). This is mediated primarily by direct lipid-protein interaction with a large number of downstream effector proteins containing specific lipid binding modules, such as the pleckstrin homology domain, the FYVE (for Fablp, YOTB, Vac1p, and EEA1) zinc finger, ENTH domain (Epsin NH2-terminal homology), and PX domain. The PX domain, consisting of 100–130 amino acids, was first identified from the sequence analysis of two SH3 domain-containing cytosolic components of NADPH oxidase, p47phox and p40phox (1, 2). The majority of proteins containing the PX domain have been classified as Sorting Nexins (SNXs) (2–4), because most of them are uncharacterized proteins speculated to function in the endosomes in a similar way as sorting nexin 1 (SNX1), the founding member of SNXs. SNX1 was isolated from a yeast two-hybrid screening using the cytoplasmic tail of the epidermal growth factor receptor (EGFR) as bait (5). Searches of sequence databases with various PX domains have revealed the existence of more related proteins (3, 4). So far, about 30 SNXs have been identified in mammalian cells (2, 4). Recent studies on SNXs have uncovered the roles of several of these proteins in membrane trafficking of plasma membrane receptors. SNX1 is involved in the down-regulation of EGFR (5, 6) and can associate with platelet-derived growth, insulin, and transferrin receptors (7), as well as protease-activated receptor 1 (9).

In this report, we describe our characterization of human IRAS. IRAS was initially isolated as an imidazoline-1 receptor candidate cloned by imidazoline receptor antisera-selected (IRAS) cDNA approach (9) and was independently shown to be an interacting partner for insulin receptor substrate 4 (10). IRAS was recently reported to protect transfected PC12 cells from apoptosis (11), whereas its mouse homologue, Nischarin, which lacks the NH2-terminal PX domain, was identified as a cytosolic interacting protein for α5 integrin and shown to inhibit cell migration by inhibiting the ability of PAK1 to phosphorylate substrates (12, 13). However, the basic cellular mechanism underlying the action of human IRAS is unknown, and our biochemical and cell biological characterizations of IRAS reveal its intrinsic property to associate with the endosomal compartments. The NH2-terminal PX domain binds specifically to PtdIns-3-P and is necessary for targeting to the early/sorting and recycling endosomes, a process dependent on homo-oligomerization mediated by a downstream C-C region. Furthermore, it associates with endogenous α5 integrin and causes a redistribution of this receptor for fibronectin from the cell sur-

EGFR, epidermal growth factor receptor; IRAS, imidazoline receptor antisera-selected; GST, glutathione S-transferase; EEA1, early endosome autoantigen 1; PHS, phosphate-buffered saline; GFP, green fluorescence protein; BFA, brefeldin A; HEK, human embryonic kidney; LRR, leucine-rich repeat; C-C, coiled-coil; PAK, p21-activated kinase; aa, amino acid(s); PE, phycocyanin.

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1 The abbreviations used are: PtdIns, phosphoinositide; PtdIns-3-P, phosphoinositide 3-phosphate; PX, phox domain; SNX, sorting nexin;
face to endosomal structures. These results suggest that human IRAS could function as an endosomal SNX regulating trafficking of proteins such as integrin.

**MATERIALS AND METHODS**

**Plasmids and Recombinant Protein**—Full-length human IRAS was obtained by PCR amplification of the entire coding region from human cDNA clone KIAA0976 (kindly provided by the Kazusa DNA Research Institute), using Primers 1-1RCP1 and 1-1RCP2 (Table 1). The resulting fragment was then digested with Xhol and EcoRV, prior to ligation to the vector pDMyc-neo (14), and pre-cut with Xhol and SmaI. This led to the construction of pDmyc-IRAS. Similarly, pDmyc-IRASAPX was constructed by PCR amplification of coding region from nucleotides 870 to 4585 of IRAS using Primers 1-1RCP5 and 1-1RCP6 (Table 1) and ligated into XhoI and MluI-digested pDmycneo. pDmyc-PX was subjected to digestion with XhoI and SacI, before it was ligated to pDmycneo-PX digested with XhoI and EcoRV. The resulting fragment was subjected to digestion with XhoI and SacI-digested pXJ-GST vector (15). Point mutation was introduced into the PX domain of IRAS using PCR products from I-1RCP5/RYVV1 and RYVV1/I-1RCP6 as templates for a second round of amplification with 1-1RCP5/I-1RCP6. The resulting fragment was then digested with Xhol and SacI, before it was ligated to pDmyc-IRASAPX digested with Xhol and EcoRV; and cloned into Xhol and SmaI-digested pDMyc-neo and pEGFP-C3. As for construction of plasmids for GST fusion proteins, PCR fragment encoding PX domain was generated using I-1RCP5 as template and was digested with NcoI and SacI (position 397) before it was ligated into the bacterial expression vector pGEX-KG (Amersham Biosciences) cut with NcoI and SacI. Diluted proteins were used for transformation and fusion proteins expression. Purification of GST fusion proteins was performed as described previously (16). Synthetic oligonucleotides were ordered from Genset (Singapore). All clones were confirmed by Automated Sequencing.

**Cell Culture and Transfection**—All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal serum, and 2% bovine serum albumin, pH 7.6. The immunostained cells were viewed using a confocal laser-scanning microscope (Nikon) attached to a Radiance 2000 imaging system (Bio-Rad).

**Immunoprecipitation and Western Blot Analysis**—Transfected 293T cells were washed twice with PBS and spun down at 800 × g for 10 min. 1.2 ml of ice-cold PBS containing 0.5% Triton X-100 and complete EDTA-free protease inhibitor mixture (Roche Diagnostics GmbH) was then added to the cell and left to lyse for 15 min on ice. Cytoplasm lysates were precleared at 13,000 rpm on a tabletop centrifuge. Typically, immunoprecipitation was carried out overnight at 4 °C with 5 μg of anti-myc or anti-GFP antibody in the presence of Protein A-Sepharose CL-4B or Protein G-agarose (Amersham Biosciences), respectively. The beads were then washed six times with PBS containing 1% Triton X-100 and 150 mM NaCl. Proteins bound to beads were subjected to 4-min boiling in the presence of with Laemmli sample buffer. The proteins were resolved by SDS-PAGE and electrotansferred onto Hybond C+ nitrocellulose. Blots (Amersham Biosciences) were incubated in blocking buffer (5% skimmed milk and 10% fetal calf serum in PBS containing 0.05% Tween 20) for at least 1 h, before they were subjected to 1-h room temperature incubation with the appropriate primary antibodies, followed by three washes with PBS (PBS with 0.05% Tween 20). Antibodies bound to the beads were detected by horseradish peroxidase-conjugated anti-mouse IgG antibodies and the SuperSignal West Pico Chemiluminescence Substrate (Pierce).

**Preparation of Total Cytosol and Membrane Fractions**—Transfected 293T cells were harvested as mentioned above. 650 μl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM KAc, 5 mM dithiothreitol) containing 1 mM phenylmethanesulfonyl fluoride and complete protease inhibitor mixture (Roche Diagnostics) were added. 10 strokes of homogenization were applied to the ice-cold cells, using a 27-gauge needle attached to a 1-ml syringe. Unlysed cells and nuclei were removed by centrifugation at 4500 rpm twice, and the pre cleared lysates were spun at 85,000 × g for 45 min. The membrane pellet was resuspended in 50 μl of lysis buffer. Equal amounts of total cytosol and total membrane fractions were loaded into a 5% SDS-PAGE. Anti-GRP78 and anti-β-actin were used as markers for cytosol and membrane fractions, respectively.

**Protein/Lipid Overlay Assay**—The protein/lipid overlay assays were performed as described previously (18). Lipid Strips™ purchased from Echelon, Inc. (Salt Lake City, UT) were pre-blocked overnight at 4°C with GST-PX domain fusion proteins (10 μg/ml) in PBST (0.05% Tween 20) with 5% bovine serum albumin (Sigma-Aldrich). The next day, the strips were washed 6 times in PBST for 10 min each, and then incubated with goat polyclonal anti-GST (Amersham Biosciences) at room temperature, after which they were washed in PBST for 6 times again. The bound proteins were detected with a horseradish peroxidase-conjugated anti-goat antibody at a dilution of 1:1000. The strips were finally subjected to 12 washes in PBST before they were visualized using ECL-SuperSignal West Pico chemiluminescence substrate (Pierce).

**Flow Cytometric Analysis**—A431 cells, grown and transfected with either pEGFP-C3 vector or pEGFP-IRAS, were starved for 4 and 5–7 days.
washed twice with PBS, before they were harvested. $1 \times 10^6$ cells were incubated on ice with 20 μl of R-PE-conjugated CD49e. Cells were washed twice with ice-cold PBS before they were subjected to flow cytometry and analyzed using FACSVantageSE system (BD Biosciences) equipped with a 488-nm argon laser.

**RESULTS**

**Human IRAS Has an NH$_2$-terminal PX Domain That Contributes to Its Membrane Association**—SNXs are characterized by the presence of a PX domain (4, 5). Some SNXs also possess one or more coiled-coil regions or other domains (3, 5). One potential SNX, a PX domain-containing protein, is imidazoline-1 receptor candidate protein or IRAS (5, 10, 12). Sequence comparison shows that the amino acid sequence of IRAS (12) is about 80% identical to that of a previously identified mouse Nischarin (1). Fig. 1A illustrates the presence of an additional 244 amino acids in the NH$_2$ terminus of the human protein in

**Fig. 1.** The NH$_2$-terminal extension of human IRAS contains a Phox (PX) domain. A, the amino acid sequences of human IRAS and mouse Nischarin are aligned. Identical or conserved residues are shown in yellow. The pink box indicates the PX domain, while the second boxed region indicates the integrin α$_5$ binding domain. Dashed lines indicate region missing in each protein. B, schematic representation of various domains in human (KIAA0975) and mouse (AF315344) IRAS predicted by the SMART (Simple Modular Architecture Research Tool) program (21). PX refers to the PX domain; LRR refers to leucine-rich repeats; CC refers to coiled-coil region; and α5-BD refers to the α$_5$ integrin binding domain. Numbers represent amino acid positions. Schematic representation of a human IRAS mutant (IRASΔPX) with truncation of the PX domain is included in this diagram. C, the PX domain of human IRAS is required for targeting to punctate structures in mammalian cells. A431 cells were transfected with myc-IRAS or myc-IRASΔPX expressing constructs. As shown, full-length myc-IRAS resides to the punctate vesicular structures, whereas IRASΔPX exhibits cytosolic distribution. Bar, 10 μm. D, human IRAS exists in both cytosolic and membrane pools. Lysates of cells expressing myc-IRAS (lanes 1 and 2) or myc-IRASΔPX (lanes 3 and 4) were fractionated into cytosolic (C) (lanes 1 and 3) and membrane (M) (lanes 2 and 4) fractions. Approximately 30% of myc-IRAS is associated with membranes and the rest in the cytosolic fraction, while myc-IRASΔPX is detected only in the cytosol. The fractions were resolved by SDS-PAGE and subjected to Western blot analysis for detection of the myc-tagged proteins (upper panel); GRP78 (luminal protein of the endoplasmic reticulum serving as a control for membrane fraction (38)) (middle panel) and β-actin (cytosolic protein control) (lower panel).
brane association and vesicular distribution of IRAS is dependent on its cytosolic fraction. These observations suggest that the membrane fractions, whereas the full-length protein is found in both the cytosol as well as membrane fractions were prepared from 293T cells expressing full-length and myc-tagged IRAS, and IRAS is important for binding to membranes, total cytosol from human IRAS. We made constructs encoding the conserved sequence of the PX domain: IRAS, as well as one that excludes the NH2-terminal extension, indicating that endogenous Nischarin has a larger size than the polypeptide encoded by the Nischarin clone (1). It is therefore important to examine IRAS in the context of intact protein and to reconcile the results derived from truncated IRAS in the new context. Based on the fact that PX domain mediates interaction with endosomal PtdIns-3-P (3, 5), we have first tested the importance of the additional NH2 terminus in subcellular distribution of human IRAS. We made constructs encoding the full-length IRAS from KIAA0975, as well as one that excludes the conserved sequence of the PX domain: IRASΔPX (Fig. 1B). Fig. 1C shows the subcellular distribution of the full-length myc-tagged IRAS, and IRASΔPX. Immunofluorescence of the full-length protein exhibits a vesicular staining, whereas the PX domain-deleted version has a cytosolic staining, as does the mouse Nischarin (1). To further demonstrate that the PX domain is important for binding to membranes, total cytosol and membrane fractions were prepared from 293T cells expressing full-length and ΔPX forms. Fig. 1D shows that the full-length protein is found in both the cytosol as well as membrane fractions, whereas ΔPX could only be detected in the cytosolic fraction. These observations suggest that the membrane association and vesicular distribution of IRAS is dependent on the presence of a PX domain at its NH2 terminus.

Detection of Endogenous IRAS in HEK 293 Cells—The NH2 terminus of IRAS (residues 10–117) was cloned into pGEX-KG (Fig. 2A), and the bacterially expressed fusion protein was subsequently used to raise rabbit polyclonal antiserum. Western blots of cell lysates with anti-IRAS antibody (Fig. 2B) demonstrated the detection of a 190-kDa protein species from total lysates of untransfected (lane 1), untagged (lane 2), and myc-IRAS (lane 3) transfected 293 cells using anti-IRAS. Similarly, immunoprecipitation and detection of untransfected 293 cell lysates using anti-IRAS could pull down a protein of ~190 kDa (lane 4), which also corresponds to the protein species precipitated from both untagged (lane 5) and myc-tagged (lane 6) IRAS. Immunoprecipitation of the same lysates using rabbit control IgG (lanes 7–9) did not result in the detection of any protein species. This indicates that IRAS exists in HEK 293 cells as a 190-kDa protein, which corresponds to the predicted size of the full-length IRAS.

Human IRAS Is Enriched in the Early/Sorting and Recycling Endosomes—To investigate in which subcellular compartments human IRAS resides, HEK 293 cells (Fig. 3A, panels A, D, G, J, and M) were double-labeled with rabbit anti-IRAS with the following markers: EEA1 and SNX-2 (early/sorting endosomes; Fig. 3A, panels B and E), transferrin receptor (sorting and recycling endosomes, panel H); LAMP1 (late endosomes and lysosomes; panel K) and GM130 (cis-Golgi; panel N). Fig. 3A indicates that endogenous IRAS is localized in the early endosomal compartment, as indicated by EEA1 (panel C) and SNX2 (panel F) as well as recycling endosomal compartments, as indicated by internalized transferrin receptor (panel I). IRAS does not seem to co-localize with late endosome/lysosomal markers LAMP1 (panel L) and GM130 (panel O). To further support our observations on the subcellular localization, we transfected HeLa cells (which are flatter and larger than HEK 293 cells) with pDmyc-IRAS (panels A, E, I, J, M, and Q) and double-labeled myc-IRAS with the following markers: EEA1 and SNX-2 (early/sorting endosomes; panels B and F), transferrin receptor (sorting and recycling endosomes, panel J); LAMP1 (late endosomes and lysosomes, panel N), and GM130 (cis-Golgi, panel R). Fig. 3B shows that a significant population of myc-IRAS (panels C and G) was localized in the same compartment as EEA1 and SNX2, whereas some of the IRAS proteins are in the same structures as the internalized transferrin receptor, which represents the recycling endosomes (panel K). myc-IRAS seems to be best co-localized with SNX2 (panel H).
On the other hand, poor co-localization with late endosome/lysosomal marker LAMP1 (panel O) was observed, whereas IRAS and GM130 seemed to be in different compartments (panel S). These observations suggest that a significant amount of myc-IRAS resides in early/sorting and recycling endosomes. Because some myc-IRAS is also localized to vesicular structures that are not marked by any of these endosomal/lysosomal markers, myc-IRAS may also reside in other domains/structures of the endosomes.

Endosomal Targeting of Human IRAS Is Both PtdIns-3-P-dependent and PX Domain-dependent—SNXs have been extensively demonstrated to target to PtdIns-3-P-enriched early endosomes (3), facilitated by the capacity of PX domain to bind phosphoinositol phosphates. To test whether the endosomal targeting of IRAS is PtdIns-3-P-dependent, A431 cells transfected with pDmyc-IRAS were treated with either 100 nM wortmannin (panels D–F), or 10 μg/ml brefeldin A (BFA) (panels J–L), both for 1 h at 37 °C. Wortmannin acts by inhibiting Class III PtdIns 3-kinases, which are responsible for generating PtdIns-3-P, whereas BFA reversibly disassembles the Golgi complex by blocking ARF-specific guanine nucleotide exchange proteins. Fig. 4A shows that, in the presence of wortmannin, myc-IRAS exhibited a cytosolic staining (panel D), indicating that inhibition of the PtdIns 3-kinase pathway affected membrane association of IRAS. The effect of wortmannin was reflected by a significant loss of EEA1 endosomal staining (panel E). BFA on the other hand, had no effect on the staining pattern of IRAS (panel J), although BFA had caused the disruption of the Golgi network, as indicated by GM130 (panel P). As controls, A431 cells were treated in a similar fashion, except that the vehicles: Me2SO for wortmannin (panels A–C) and methanol for BFA (panels G–I) were used instead. The binding of IRAS to PtdIns-3-P is likely caused by the presence of an active PX domain. The structure of the PX domain of p40phox has been resolved, and the region that binds the phosphoinositol head group was formed by a β3/α2-loop (18). Sequence alignment of IRAS with other SNXs (5) indicates that the highly conserved stretch of Arg-Arg-Hyd-Ser-(Asp,Glu)-Phe along the β3α2 region of the PX domain could also be found in the PX domain of IRAS, except that in place of the first Arg of the consensus sequence, is an His residue (Fig. 4B). Mutation of the Arg and Tyr residues into two Val residues destroyed the punctate staining of IRAS, resulting in diffuse cytosolic distribution. Taken together, we conclude that the endosomal targeting of IRAS requires the presence of PX domain-mediated interaction with endosomal PtdIns-3-P.

The PX Domain of Human IRAS Is Able to Bind PtdIns-3-P but Unable to Target to Endosome, Unless It Is Dimerized—Our above results suggest that the PX domain of IRAS is likely to bind PtdIns-3-P. We used the protein-lipid overlay assay (17), which involves probing blots of nitrocellulose membranes pre-spotted with the respective lipids (Fig. 5A) with the target protein. Different regions of IRAS were expressed as recombinant GST fusion proteins. GST-PX-CC and GST-PX consist of residues 1–695 and residues 1–133 of IRAS, respectively. GST-PX/RY-VV) on the other hand, consists of a similar region as GST-PX, except that Arg49 and Tyr50 have been mutated into two Val residues. A concentration, 10 μg/ml of each of the GST-tagged SNX3, GST-PX-CC, GST-PX, GST-PX/RY-VV), and GST protein, was used in this experiment. GST-SNX3, shown previously to bind PtdIns-3-P (19) was used as a positive control, whereas GST protein was included as a negative control. As can be seen from Fig. 5A, GST-SNX3, GST-PX-CC, and GST-PX bound specifically and exclusively to PtdIns-3-P. No binding to any of the lipid species was observed for GST protein as well as GST-PX(RY-VV), implying that an active PX domain was the minimal requirement for binding to PtdIns-3-P.

We then proceeded to test whether the PX domain alone was sufficient in targeting to endosomes, by cloning it into mammalian expression vectors containing GST (pXGST) and myc-tag (pDmycense), respectively. A431 cells transfected with pXGSTPX (Fig. 5B, panel A) exhibited an endosomal staining that coincided significantly (panel C) with the immunostaining of EEA1 (panel B). However, the same PX domain, when expressed as a myc-tagged recombinant protein (panel G), was unable to target to endosomes (panel I). It was unlikely that GST per se of GST-PX could target to endosomes, because the GST tag by itself (panel D), exhibited a cytoplasmic staining. This led us to suspect that the GST tag had a property that contributed to the increased ability of the PX domain to bind PtdIns-3-P on early endosomes.

The Coiled-coil Region of Human IRAS Is Important for Endosomal Targeting—One possible property that GST exhibits is its ability to dimerize. GST has recently been demonstrated to exist in its native state as a dimer (20). This led us to suspect that GST tag at the N-terminal of GSTPX could dimerize, bringing two molecules of PX domain in close proximity, thereby increasing the avidity of PtdIns-3-P binding. Sequence examination of IRAS using the SMART motif prediction program (21, 22) suggests the presence of a stretch of five leucine-rich repeats (LRRs) (residues 286–401) and a coiled-coil (C-C) region (residues 634–695) (Fig. 1B). Expression of a...
series of clones that encode different lengths of the IRAS (Fig. 6) revealed that the presence of the PX domain alone (panel A) or the sequence thereafter (panel B) were insufficient for localization to the vesicular compartments. Inclusion of the five LRRs, previously proposed to be protein-recognition motifs, (23), did not seem to facilitate vesicular localization (panel C). It was not until the coding region for the C-C domain was included, that we started observing endosomal staining (panel D), in a manner similar to that of the full-length IRAS (panel F). Panel E indicates a similar endosomal staining of A431 cells transfected with a construct pmyc-PX-a5-BD that expresses amino acids 1–826 of human IRAS. The stretch of residues between 190 and 826 of human IRAS is likely to contain the α5 integrin-binding site previously identified for mouse Nischarin (Fig. 1A, second boxed region) (1). These observations led us to conclude that a combined action of the PX domain (in recognition of phosphoinositides) and the C-C region are necessary for IRAS to target to the endosomes. These results agree with other observations, where the C-C region in SNXs are essential. For instance, in SNX1 deletion of a predicted C-C region abolished vesicle localization, indicating that this helical domain is necessary for SNX1 localization (7). In SNX16, the C-C region is found to be involved in homo-oligomerization and is necessary for distribution in later endosomal structures (24).

Human IRAS Is Likely to Homo-oligomerize via Its Coiled-coil Region—To further support our hypothesis that the C-C region of IRAS could facilitate dimerization of the protein, we cloned the coding region of IRAS from residues 120 onward, into pEGFP-C3, thereby replacing the PX domain with the green fluorescent protein (GFP). Constructs pmyc-IRAS and pmyc-PX-IRASΔPX were individually or co-transfected into 293T cells. Fig. 7 illustrates the co-immunoprecipitation of myc-tagged IRAS and GFP-tagged IRASΔPX. In this study, the ability to co-immunoprecipitate was used as an indication of homo-oligomerization of IRAS. Western blots of cell lysates inputs with anti-myc (lane 1) and anti-GFP (lane 3) antibodies demonstrated the expression of myc-IRAS and GFP-IRASΔPX, respectively, as well as equal amounts of each protein expressed in co-transfected cells (lane 2). Immunoprecipitation of myc-IRAS using anti-myc could pull down a protein species of ~150 kDa, which could be recognized by anti-GFP, from co-transfected cells (lanes 5). At the same time, immunoprecipitation of GFP-IRASΔPX using anti-GFP led to the detection of 190-kDa band with anti-myc antibodies. The result suggests that IRAS is able to homo-oligomerize, most likely through the C-C domain. This mode of interaction is independent of the PX domain, which is deleted in GFP-IRASΔPX. It is tempting for us to suggest that the function of the C-C domain, in the context of homodimer formation, is to bring two copies of PtdIns-3-P recognition motifs (the PX domains) in close proximity, thereby facilitating the binding of IRAS to endosomes. The function of this region could be changed when the PX domain is fused to dimerizing GST but not to the myc tag.

Human IRAS Associates with α5 Subunit of Integrin and Causes Its Redistribution from Plasma Membrane to Intracellular Vesicles—Because mouse Nischarin has been implicated to play an important role in fibroblast migration and regulation of actin cytoskeleton by binding to the α5 subunit of integrins, we decided to test if human IRAS, being so highly homologous (except for the presence of an NH2-terminal PX domain), can associate with α5 subunit integrin and affect its trafficking. Integrins are fibronectin receptors that consist of a heterodimer of α and β subunits (25). Studies on the localization of αβ3 during polymorphonuclear neutrophil migration suggest that fibronectin receptors are endocytosed and trafficked through an endocytic recycling compartment before being recycled back out to the cell surface (26). Because our results so far indicate that IRAS is a sorting nexin that is localized to early/sorting and recycling endosomes, it is possible that it may play a part in the recycling/trafficking process of α5 integrin. First, we tested if IRAS is associated with α5 integrin in vivo (Fig. 7A). We transiently transfected 293T cells with myc-tagged IRAS (lane 1) and performed immunoprecipitation of the lysate using an antibody raised against the α5 subunit of integrin, suggesting that IRAS could interact with the endogenous α5. We then proceed to test whether subcellular localization of α5 integrin in mammalian cells would be altered by overexpression of IRAS. We observed that when A431 cells were seeded at a low ~40% density, while in the presence of fetal calf serum containing fibronectin and various growth factors, the majority of α5 integrin was located at the cell surface (Fig. 7B, untransfected cells) as detected by a monoclonal antibody raised against human α5 integrin. Significantly, in cells overexpressing IRAS, surface labeling for α5 integrin was reduced with a shift of α5 integrin into the punctate structures marked by IRAS, suggesting that high levels of IRAS may cause the redistribution of α5 integrin from the cell surface to the endocytic compartments. It is possible that interaction of IRAS with α5 integrin may play a regulatory role in the endocytic trafficking of this fibronectin receptor by sequestration of endogenous α5 integrin causing a reduction in the number of surface receptors used for anchorage during cell migration (1, 13). The levels of plasma membrane α5 integrin in A431 cells overexpressing either GFP or GFP-IRAS were assessed by flow cytometry. Cells were kept on ice to prevent internalization of the cell surface α5 integrin. These fibronectin receptors were detected by immunofluorescence using a PE-conjugated anti-α5 integrin antibody. The amount of cell-surface α5 integrin represented by PE positivity was significantly reduced by GFP-IRAS (Fig. 8C, bottom) with a mean value of 162) as compared with the mean value of 238 in GFP expressing cells (Fig. 8C, top). This result indicated that the observations of reduced staining of cell surface α5 integrin in Fig. 8B could be due to overexpression IRAS.

**DISCUSSION**

In this report, we demonstrate for the first time that the human IRAS (12) exhibits properties of a typical SNX, providing a cellular and mechanistic basis for its participation in various cellular processes. In contrast to mouse Nischarin (1), which likely represents a truncated protein, IRAS has an extra stretch of 244 amino acids at the NH2 terminus (12). The first half of this NH2-terminal extension is characterized as a PX domain based on amino acid sequence homology (5), the hallmark of an SNX. The PX domain was first identified in 1996 as a stretch of ~120 amino acids present in two components (p40phox and p47phox) of the phagocyte NADPH (phox) complex (2) and is a PtdIns-4-P-binding module (5, 27–30). The presence of a PX domain in human IRAS has prompted us to investigate its cellular and biochemical properties so that its participation in cellular functions could be studied and reconciled in the right context. A significant amount of IRAS was targeted to sorting and recycling endosomes marked by EEA1, SNX2, and transferrin receptor, with an additional amount present in vesicular structures that are not marked by known endosomal/lysosomal markers. This suggests that IRAS has a broader distribution in the endosomal system, a point that warrants additional studies. Based on this finding, we have focused on the molecular mechanism underlying this endosomal targeting of human IRAS.

A combined action of PX domain-mediated interaction with PtdIns-3-P on the endosomes and homo-oligomerization medi-
FIG. 3. Human IRAS is targeted to early/sorting and recycling endosomes but not to late endosomes/lysosomes or the Golgi apparatus. A, human embryonic kidney 293 cells grown on coverslips were processed for double-labeling using anti-IRAS antibodies and anti-EEA1 (A–C), anti-IRAS and anti-SNX2 (D–F), anti-IRAS and anti-transferrin receptor (G–I), myc-tag and LAMP1 (J–L), or myc-tag and GM130 (39) (M–O). As shown, a significant amount of IRAS co-localizes with early/sorting endosomes represented by EEA1 and SNX2 and the recycling endosomes marked by TfR. Bar, 10 μm. B, HeLa cells were transfected with myc-IRAS expressing construct. Cells were processed for double-labeling using antibodies against the myc tag and EEA1 (A–D), myc tag and SNX2 (E–H), myc tag and transferrin receptor (I–L), myc-tag and LAMP1 (M–P), or myc-tag and GM130 (Q–T). As shown, a significant amount of IRAS co-localizes with early/sorting endosomes represented by EEA1 and SNX2 and the recycling endosomes marked by TfR. Bar, 10 μm.
ated by its coiled-coil region appears to be responsible for the endosomal distribution of IRAS. The role of PX domain was supported by our demonstration that it binds specifically PtdIns-3-P. PtdIns 3-P is produced by Vps34p/class III PtdIns 3-kinases and operates via the PtdIns 3-P-binding proteins, such as EEA1. The presence of a stretch of five leucine-rich-repeats (LRRs) did not seem to have a direct effect on endosomal targeting, because a mutant containing the PX domain and LRRs could not be delivered to the endosomes. The primary function of LRRs is not known and might be for the provision of a versatile structural framework for the formation of protein-protein interactions between IRAS itself or between IRAS and its potential interacting partners (23). Inclusion of the coiled-coil region is essential for endosomal targeting. Because the PX
domain of IRAS dimerized by fusion with GST is efficiently targeted to the endosomes and a mutant IRAS without the PX domain can form homo-oligomer with IRAS, it is reasonable to conclude that one primary role of other regions (especially the coiled-coil region) is likely to mediate homo-oligomerization. Our results do not provide evidence for a role of IRAS to function as a cell surface receptor for imidazoline but are consistent with the remaining possibility that it could act as an

**Fig. 4.** The endosomal association of IRAS is disrupted by treatment with Wortmannin but unaffected by brefeldin A. A431 cells transfected with myc-IRAS expressing construct were treated for 1 h at 37 °C with Me2SO (A–C); 100 nM wortmannin (panels D–F); methanol (panels G–I), or 10 μg/ml brefeldin A (BFA) (panels J–L). Upon paraformaldehyde fixation and permeabilization with 0.2% Triton X-100, the treated cells were processed for double-labeling using antibodies against myc tag and EEA1 (panels A–F) or myc tag and GM130 (G–L). As shown, the endosomal labeling of myc-IRAS (together with that of EEA1) is lost upon treatment with wortmannin (panels D–F), resulting in diffuse cytosolic labeling. The cis-Golgi (marked by GM130) was redistributed by BFA to adopt a labeling characteristic of spotty ER exit sites (panel K), the endosomal labeling of myc-IRAS in the same cell (panel J) was unaffected by BFA. Bar, 10 μm. B, an intact PX domain is required for endosomal targeting. The conserved stretch of residues Arg-Arg-hydrophobic-Ser-Asp/Glu-Phe along the β2α2-loop of a PX domain can also be found in human IRAS. A431 cells were transfected with constructs expressing myc-IRAS and a myc-IRAS mutant carrying Arg49→Val and Tyr50→Val mutations. As shown, myc-IRAS exhibits endosomal staining, whereas myc-IRAS(RY-VV) mutant has a diffuse cytoplasmic labeling. Bar, 10 μm.
intracellular binding protein for imidazoline or as a subunit of a membrane receptor.

Like SNX1, PX domain (residues 1–133) of IRAS is not sufficient for endosomal targeting, unless the putative C-C domain (residues 630–695) is included. When a predicted C-C region in the COOH terminus of SNX1 was deleted, the mutant fails to be targeted to vesicular endosomes (7). Similar observations had been made for proteins containing another type of PtdIns-3-P binding domain: the FYVE zinc finger. The FYVE domain of early endosome autoantigen (EEA1) is essential, but alone not sufficient for endosomal targeting. The presence of the C-C domain is crucial for its efficient localization to early endosomes (31). Similarly, both FYVE and the C-C domains are needed to target the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) specifically to early endosomes (32).

It is interesting to notice that EEA1 has been shown to exist as a homodimer facilitated by the presence of C-C region (33), and this form of dimerization may increase the avidity of EEA1 for PtdIns-3-P-containing endosome membranes (34).

Our results suggest that IRAS may exist as a homodimer through interaction between the C-C regions, whereas its PX domain, isolated as a monomer, is not sufficient for endosomal targeting. This behavior is different from that reported for SNX3 (19). SNX3, which consists almost entirely of a PX domain, could bind to early endosomes without the requirement for a C-C domain (19). We have thus examined the conserved stretch of residues along the z-loop of the PX domain carefully (3, 5). The conserved stretch of residues makes up the major part of the pocket that binds the head group of PtdIns-3-P. The second Arg in this sequence context forms the most extensive interaction with the D3 phosphate of the bound PtdIns-3-P (18). The sequence for SNX3 is Arg68-Arg69-Tyr70...
Ser\textsuperscript{71}-Asp\textsuperscript{72}-Phe\textsuperscript{73}, which fits perfectly with the consensus sequence of Arg-Arg-hydrophobic-Ser-Asp/Glu-Phe. The sequence of IRAS on the other hand, is "imperfect": His\textsuperscript{48}-Arg\textsuperscript{49}-Tyr\textsuperscript{50}-Ser\textsuperscript{51}-Asp\textsuperscript{52}-Phe\textsuperscript{53}. In place of the first Arg, is a His, an uncharged amino acid at physiological pH. Presumably, a positively charged Arg\textsuperscript{68} in the PX domain of SNX3 would be better in creating a more positively charged environment for stronger binding of D\textsubscript{3} phosphate to the crucial Arg\textsuperscript{69}. This form of perfect complementation would not be present in the PX domain of IRAS but could be potentially strengthened by dimerization. Our speculation for this scenario of endosomal targeting of IRAS is that the weaker affinity of its PX domain (monomer) for endosome is probably an important property for the role of IRAS in trafficking cell surface receptors. The necessity for homo-oligomerization of two molecules of IRAS, to increase the association of dimerized PX domains with PtdIns-3-P-enriched endosomes, would allow for a more delicate control over its function in regulating trafficking process of other proteins.

SNX1, the founding member of SNXs, was isolated from a yeast two-hybrid screen using the cytoplasmic tail of the epidermal growth factor receptor (EGFR) as bait, and it interacted specifically with the lysosomal targeting sequence of EGFR (6). SNX1 is involved in down-regulation of activated cell surface EGFR through degradation in lysosomes. Amino acid sequence homology was noticed in a region (now known as the PX domain) between SNX1 and Mvp1, a yeast protein known to target hydrolases to yeast vacuoles (35). SNX1 has also been reported associate with platelet-derived growth, insulin, and transferrin receptors (8), as well as protease-activated receptor 1 (9). SNX6 associates with the transforming growth factor \(\beta\) (TGF-\(\beta\)) family of receptor serine-threonine kinase (36). The results reported here suggest that human IRAS is likely to function as a sorting nexin in the endosomal pathway, although its precise function in membrane trafficking is currently unclear. However, the demonstration of an interaction between mouse homologue Nischarin and \(\alpha_\text{5}\)-integrin (1, 13, 37) implies that human IRAS could regulate the trafficking process of integrin, the fibronectin receptor. In support of such a possibility is our demonstration that PX domain-containing IRAS remains capable of interaction with \(\alpha_\text{5}\)-integrin, suggesting that the NH\textsubscript{2}-terminal extension does not affect the property of \(\alpha_\text{5}\)-integrin-interacting region. In addition, under the condition in which \(\alpha_\text{5}\)-integrin is predominantly targeted to the plasma membrane, overexpression of myc-IRAS causes a shift of \(\alpha_\text{5}\)-integrin into intracellular structures marked by IRAS. Also, quantitative

![Fig. 6. The PX domain and coiled-coil region of human IRAS are required for endosomal targeting.](image)

![Fig. 7. Homo-oligomerization of human IRAS.](image)
**Fig. 8.** Human IRAS interacts with endogenous \( \alpha_5 \) integrin and affects the surface expression of \( \alpha_5 \) integrin.

**A**, untransfected or pDmyc-IRAS-transfected 293T cells were lysed and subjected to direct analysis by SDS-PAGE (lanes 1–2) or to immunoprecipitation with anti-myc antibody (lanes 3–4). Upper panel refers to detection of myc-IRAS by anti-myc antibody by Western blot analysis, whereas the lower panel refers to detection of endogenous \( \alpha_5 \) integrin with anti-\( \alpha_5 \) antibody. As shown, endogenous \( \alpha_5 \) integrin could be immunoprecipitated together with myc-IRAS (lane 3). Co-immunoprecipitation of \( \alpha_5 \) integrin by anti-myc antibodies was not observed in control cell (lane 4).

**B**, expression of human IRAS causes a redistribution of cell surface \( \alpha_5 \) integrin to intracellular vesicular structures marked by IRAS. A431 cells grown at 40% confluency were transfected with pDmyc-IRAS and were subsequently processed for double-labeling using antibodies against the myc tag and the endogenous \( \alpha_5 \) integrin. As shown, \( \alpha_5 \) integrin was detected predominantly on the cell surface (particularly the cell boundary) in cells that do not express myc-IRAS. In cells expressing myc-IRAS, bright labeling of \( \alpha_5 \) integrin in endosomes marked by IRAS was detected and the strong labeling of the cell boundary was significantly reduced.

**C**, A431 cells were transfected with pEGFPC3 as a control or pEGFPC3IRAS, and analyzed for cell-surface levels of \( \alpha_5 \) integrin using flow cytometry. Cell-surface \( \alpha_5 \) integrin (PE positivity) was measured on untransfected A431 cells (not shown), or cells expressing GFP or GFP-IRAS. The histogram shows the \( \alpha_5 \) integrin levels (PE log scale) on GFP (top) and GFP-IRAS (bottom) cells against relative number of events. The mean PE values for GFP and GFP-IRAS are indicated by green and red lines, respectively.
analysis by flow cytometry had indicated a 30% reduction in the amount of cell surface $\alpha_\delta$ integrin during IRAS overexpression. Taken together, our data support an interaction between IRAS and $\alpha_\delta$ integrin but also indicate that IRAS interferes with the trafficking of $\alpha_\delta$ integrin by sequestration. The mouse homologue Nischarin has recently been shown to interact with members of the PAK family of kinases, such as PAK1, in its activated conformation (37). Interaction with Nischarin strongly inhibits the ability of PAK1 to phosphorylate phosphoamides. This effect on PAK kinase activity closely parallels the ability of Nischarin to inhibit cell migration.

Because IRAS interacts with several substrates of insulin receptor (11), it could also recruit these substrates to endosomal membranes so that they could serve as more effective substrates for endosomal insulin receptor. Alternatively, IRAS could act together with these substrates to regulate the trafficking/signaling process of insulin receptor. Further studies along these lines will provide precise insights into the functional aspects of IRAS.

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54782 Human Nischarin/IRAS Targeting
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