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SUMMARY

The role of clathrin in the sorting of proinsulin to secretory granules, the formation of immature granules and their subsequent maturation is not known. To this end, primary rat pancreatic β-cells were infected with a recombinant adenovirus co-expressing the Hub fragment, a dominant-negative peptide of the clathrin heavy chain and enhanced green fluorescent protein (EGFP as a marker of infected cells). A population of cells expressing the highest levels of EGFP (and thus Hub) was obtained using a fluorescence-activated cell sorter (FACS). Control cells were infected with an adenovirus expressing EGFP alone. By immunofluorescence, control cells showed intense staining for both clathrin light chain and proinsulin in a perinuclear region. In cells expressing high levels of Hub, the clathrin light-chain signal was faint and diffuse in keeping with its displacement from membranes. There was, however, no detectable effect of Hub expression on proinsulin staining or disposition within the cell. Proinsulin sorting and conversion, and the fate (release and/or degradation) of insulin and C-peptide, was studied by pulse-chase and quantitative reverse phase HPLC. In both Hub-expressing and control cells, >99% of all newly synthesized proinsulin was sorted to the regulated pathway and there was no effect of Hub on proinsulin conversion to insulin. In presence of Hub there was, however, a significant increase in the percentage of C-peptide truncated to des-(27-31)-C-peptide at early times of chase as well as more extensive degradation of C-peptide thereafter. It is concluded that clathrin is not implicated in the sorting or processing of proinsulin or in regulated exocytosis of secretory granules. These results confirm a role for clathrin in the removal of proteases from maturing granules, thus explaining the increased truncation and degradation of C-peptide in cells expressing Hub.

Key words: Clathrin, Proinsulin, Insulin, C-peptide, Trafficking, Regulated secretion

INTRODUCTION

Clathrin is involved in many intracellular vesicular trafficking pathways. It decorates the cytosolic face of membrane regions destined to bud and form (clathrin-coated) vesicles that become uncoated before fusing with their target membrane, most typically the endosomal compartment (for recent reviews see Hirst and Robinson, 1998; Kirchhausen, 1999; Kirchhausen, 2000; Le Borgne and Hoflack, 1998; Schmid, 1997). In highly specialized neuroendocrine cells equipped with the regulated secretory pathway, it has long been recognized that both those regions of the trans-Golgi network destined to bud off in the form of nascent dense-core granules (Orci et al., 1985), as well as the earliest (immature) granules themselves, carry a discrete coat of clathrin (Orci, 1982; Tooze and Tooze, 1986). Immature granules are notable in that their clathrin coat, unlike that of other clathrin-coated vesicles, is seen by electron microscopy to be discontinuous (Orci, 1982; Orci et al., 1984b). The adapter protein AP-1 has been implicated in clathrin coating of immature granules in other regulated secretory cell types (Dittie et al., 1996). In the pancreatic β-cell, the immature clathrin-coated granule has been shown to be mildly acidic, rich in proinsulin but poor in insulin (Orci et al., 1994). As granules mature, so they become progressively more acidic (Orci et al., 1994). This allows for activation of the proinsulin conversion endoproteases with ensuing conversion of the prohormone to insulin and C-peptide (Orci et al., 1987; Rhodes and Halban, 1987). Contemporaneously, the clathrin coat is lost, and there is morphological evidence to suggest that this may reflect the budding of bona-fide clathrin-coated vesicles from maturing granules (Orci, 1982).

Although the precise role of clathrin in the regulated secretory pathway of β-cells, and indeed any other neuroendocrine cell type, remains obscure, two possibilities merit consideration. Clathrin may be implicated in sorting events within the trans-Golgi network (TGN), allowing proinsulin and/or other proteins important in the regulated pathway (including the conversion endoproteases) to be delivered to nascent granules, or it could have a role in the formation of such granules. Alternatively, and possibly additionally, clathrin may be implicated in the formation of vesicles budding from maturing granules (Arvan and Castle, 1998). There are two variants of clathrin light, LCa and LCb. There is a predominance of LCb in cells with a regulated secretory pathway (Acton and Brodsky, 1990). This suggests that clathrin may play a specific and perhaps unique role in this pathway.

Clathrin forms triskelions, consisting of clathrin heavy and light chains (Brodsky, 1988; Kirchhausen, 2000). The Hub fragment (comprising the C-terminal third of the heavy chain) mimics the central portion of the triskelion and can bind light chains to form nonproductive heteropolymers incapable of coating membranes (Liu et al., 1995). Expression of Hub in living cells leads to depletion of clathrin light chain and disruption of clathrin function (Liu et al., 1998). The Hub fragment can thus be considered a dominant-negative mutant clathrin heavy chain, and its expression in HeLa cells led to the inhibition of clathrin-mediated membrane transport (Liu et al., 1998). Such a dominant-negative strategy for the study of clathrin function in mammalian cells complements earlier studies in clathrin-deficient mutant organisms including yeast (Payne et al., 1987; Payne and Schekman, 1985; Payne and Schekman, 1989; Seeger and Payne, 1992; Silveira et al., 1990). Hub has now been expressed at high levels in primary rat pancreatic β-cells using a recombinant adenovirus, in order to study the importance of clathrin for proinsulin trafficking and processing, as well as the regulated secretion of proinsulin and its conversion products, insulin and C-peptide.

MATERIALS AND METHODS

Materials

Tissue culture reagents were obtained from Gibco (Gibco Life Technologies, AG Basel, Switzerland). All others reagents were purchased from Sigma (Sigma, St Louis, MO) unless stated otherwise.

Preparation of recombinant adenovirus

The HindIII-BglII cDNA fragment encoding the T7-tagged Hub peptide from pCDM8T7Hub (Liu et al., 1998) was subcloned into the HindIII-BglII sites of the pTrackCMV vector (He et al., 1998) to produce the pTrack-T7Hub. The bacterial strain BJ5183 was co-transformed with 0.1 μg of pTrack-T7Hub linearized by PmeI and with 5 μg of the adenovirus DNA. HEK293 cells were transfected with the recombinant adenovirus DNA linearized by PacI to produce adenovirus expressing the T7-tagged Hub peptide (‘Hub virus’). The control adenovirus without insert (‘empty virus’) was produced the same way.

Cell culture and infection

Islets of Langerhans were isolated by collagenase digestion of the pancreas of adult male Sprague-Dawley OFA rats and the islets digested with trypsin to obtain a suspension consisting predominantly of single cells according to standard procedures (Rouiller et al., 1990). To allow them to recover from the isolation and digestion procedures, the cells were placed in plastic Petri dishes (35 mm diameter; 5×10^5 cells/dish) coated with a matrix secreted by 804G (rat bladder carcinoma cells from Desmos, San Diego, CA) and maintained in culture (DMEM, 11.2 mM glucose, 10% FCS) for 24 hours, during which time they adhered to the matrix and spread (Bosco et al., 2000). Cells were then incubated for 3 hours at 37°C in complete medium (as above) with recombinant adenovirus expressing either enhanced green fluorescent protein (EGFP) alone (empty virus) or EGFP and Hub fragment (‘Hub virus’) and then washed 2× in PBS before culture overnight in complete medium. The quantity of virus used for infection was established empirically to allow for maximum expression of EGFP without any detectable toxicity. The multiplicity of infection (moi) was in all cases less than 100, a titre shown by us in previous experiments to be nontoxic for β-cells (Irminger et al., 1996; Meyer et al., 1998). The following day, the cells were trypsinized and then purified by fluorescence-activated cell sorting (FACS using a FACStar Plus, Becton and Dickinson, Sunnyvale, CA) on the basis of their EGFP fluorescence. The sorted (fluorescent) cells were allowed to recover from the procedure for 6 hours in culture in nonadherent plastic dishes and were then plated in droplets (5×10^4 cells/75 μl droplet of complete medium) on plastic dishes covered with 804G matrix. Pulse-chase or immunofluorescence experiments were performed the following day.

Pulse-chase

Infected (sorted) cells were washed twice with a modified Krebs-Ringer bicarbonate buffer (KRB-Hepes: 134 mM NaCl, 4.8 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 2.8 mM glucose, 0.25% BSA, 10 mM Hepes pH 7.4) then incubated for 15 minutes at 37°C in KRB-Hepes containing 16.7 mM glucose, followed by 15 minutes at 37°C in the same buffer containing 1 mCi/ml [^{3}H]leucine. The cells were washed twice with KRB-Hepes and incubated for 150 minutes at 37°C with KRB-Hepes (basal secretion), followed by 1 hour at 37°C with KRB-Hepes supplemented with 16.7 mM glucose, 10 mM leucine, 10 mM glutamine, 1 mM IBMX, 10 μM forskolin and 0.1 μM PMA (stimulated secretion). The two secretion media (basal and stimulated) were centrifuged at 2000 rpm for 5 minutes to remove any cells that may have detached from the dish during incubations. The cells were extracted in 1 M acetic acid, 0.1% BSA, freeze-thawed twice to ensure complete extraction of cellular proteins, and centrifuged to remove debris (10 minutes; 12,000 rpm, microfuge).

Samples were analysed by reverse-phase HPLC using a well-established method allowing for separation and quantification of radiolabelled proinsulin, conversion intermediates, insulin, C-peptide and truncated (des-(27-31)-) C-peptide as described previously (Irminger et al., 1996; Verchere et al., 1996).

Immunofluorescence

Cells were washed twice in Dulbecco’s PBS (DPBS), then fixed in 4% paraformaldehyde for 15 minutes, washed again twice with DPBS and permeabilized for 10 minutes in DPBS plus 0.2% Triton X-100, 0.1% BSA. They were blocked for 1 hour in DPBS containing 5% BSA before incubation for 1 hour at room temperature with monoclonal anti-clathrin light chain (CON.1; BAbCO, Richmond, CA) diluted 1/200 (in DPBS 1% BSA), monoclonal anti-proinsulin (GS 4G9, the generous gift of O. Madsen, Hagedorn Research Institute, Gentofte, Denmark; this antibody recognizes proinsulin but not insulin) diluted 1/50 or polyclonal anti-rat cathepsin B (Upstate biotechnology, Lake Placid, NY) diluted 1/100. The cells were then washed twice with DPBS and incubated with a goat anti-mouse or anti-rabbit TRITC conjugated antiserum diluted 1/1000 in DPBS 1% BSA for 1 hour at room temperature in the dark, washed and mounted using Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence and direct fluorescence (EGFP) images were taken using a confocal microscope (Zeiss).

Presentation of data and statistical analysis

Data are presented as the mean±s.e.m. for ‘*n*’ independent experiments. Significance of differences between groups was evaluated using Student’s two-tailed t-test for unpaired groups ($P<0.05$ considered significant).

RESULTS

Expression of high levels of Hub affects the intracellular localization of clathrin light chain but not of proinsulin

β-cells were infected with Adeno-Hub or adenovirus expressing EGFP alone and examined (without prior sorting) by immunofluorescence following staining with antibodies to clathrin light chains (LC) and proinsulin. Cells infected with

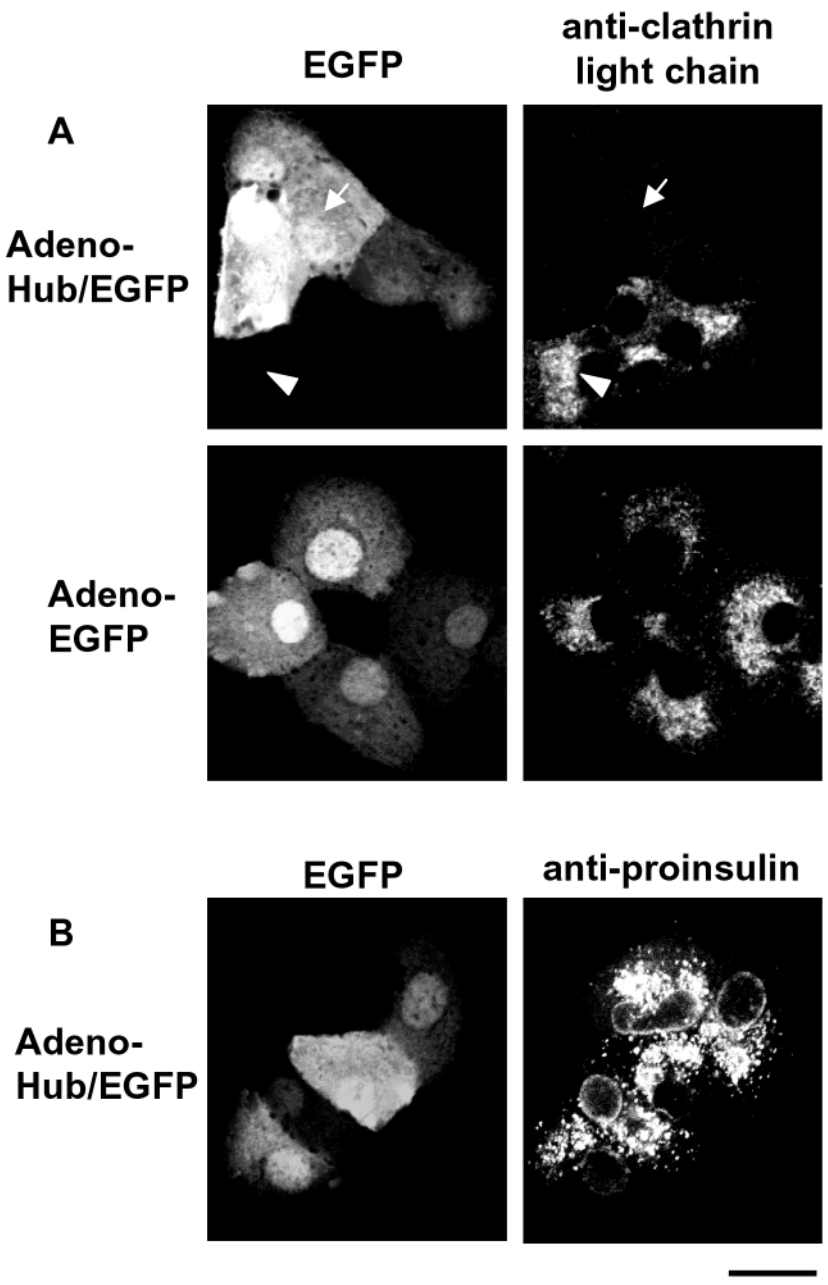

Fig. 1. Effect of Hub expression on immunolocalization by confocal microscopy of clathrin light chain and proinsulin. Rat islet cells were infected with Adeno-Hub/EGFP (a recombinant adenovirus expressing both the Hub peptide and EGFP such that the latter is a marker for Hub expression) or with Adeno-EGFP (only expressing EGFP) as indicated. The left-hand images show direct EGFP fluorescence. Right-hand images show staining for (A) clathrin light chain; (B) proinsulin. Control cells infected with Adeno-EGFP show a strong perinuclear and punctate staining for clathrin light chain regardless of the level of EGFP expression (A: lower). Expression of Hub leads to the disappearance of the perinuclear and punctate staining pattern for clathrin light chain in those cells with the highest level of expression of EGFP (and thus Hub – see representative cell marked by arrow in A: upper), whereas cells in the same cluster but expressing low or nondetectable levels of EGFP (and thus Hub) present normal clathrin light chain staining (see representative cell marked by arrowhead in A: upper). Proinsulin immunostaining (B) was unaffected by expression of Hub, with all cells presenting the same staining pattern regardless of the level of EGFP expression. Bar, 10 μm.

control virus displayed intense perinuclear staining for both clathrin LC and proinsulin (Fig. 1). There was also faint staining for LC on the plasma membrane but this was barely discernible using the confocal microscope. Expression of Hub led to a dramatic change in LC staining, which appeared as only a diffuse background staining suggestive of its redistribution to the cytosol in cells with intermediate levels of Hub, and was no longer detectable in cells with the highest levels (which is not to suggest that it was no longer present). There was no apparent effect of Hub-expression on the localization or intensity of staining for proinsulin. These results are in keeping with the effect of Hub expression on LC localization in HeLa cells, indicating that Hub was indeed acting as a dominant-negative mutant clathrin heavy chain but that this was apparently without effect on proinsulin synthesis or subcellular compartmentalization.

Use of FACS to sort cells expressing high levels of Hub on the basis of their EGFP fluorescence

The effect of Hub on clathrin LC disposition was most evident in cells expressing high levels of the peptide (for example, the cell in Fig. 1A, top panel, indicated by the arrow). In order to limit the studies to such a population of cells, they were first sorted by FACS. The Hub peptide expressed in the recombinant adenovirus carried an N-terminal T7 epitope allowing for quantification of Hub expression using anti-T7 antibodies. Given that the adenovirus co-expressed EGFP (not, it must be stressed, as a fusion protein) it follows that expression of EGFP should correlate with that of T7 and thus of Hub itself. This was seen to be the case when infected cells were examined by direct fluorescence (for EGFP) in combination with immunofluorescence (using anti-T7 and rhodamine-labelled second antibody; data not shown). Cells expressing high levels of Hub could thus be sorted by FACS on the basis of their EGFP fluorescence. Analysis of fluorescence by FACS confirmed the expected heterogeneity of EGFP expression. The sorting windows were set such as to recover only the 20% most fluorescent cells. All biochemical experiments were performed on such sorted cells. When examined for clathrin light chain immunofluorescence, they all displayed the expected phenotype, with the barely discernible diffuse staining typical for cells expressing high levels of Hub as described above. For controls, cells were infected with the adenovirus expressing EGFP alone and sorted using the same sorting parameters as for the Hub-infected cells. Cells were sorted 24 hours after infection with adenovirus and then maintained in tissue culture for a further 24 hours. This allowed cells to recover from the sorting procedure and more importantly provided sufficient

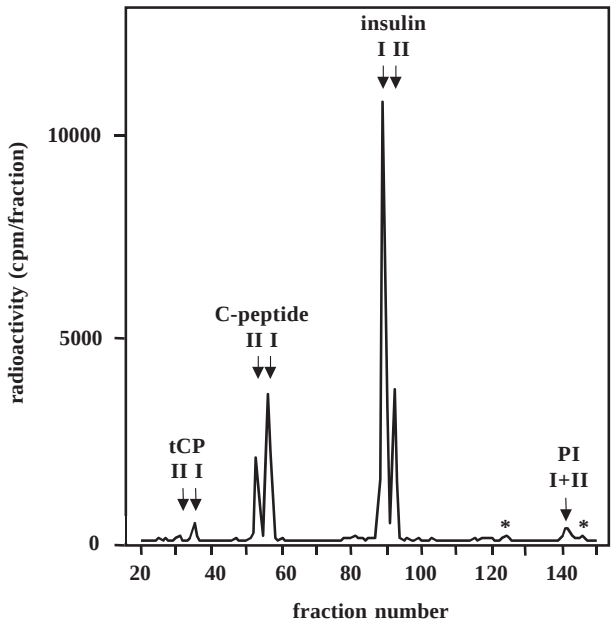

Fig. 2. Reverse phase HPLC analysis of radioactive products in islet cells. Cells were pulse-labelled and then chased for 150 minutes under basal conditions before stimulation with a cocktail of secretagogues for 60 minutes. The representative profile is for radioactive products released during this 60 minute period with elution times for the two nonallelic rat I and II proinsulins (PI) and their conversion products as indicated by the arrows. *Elution times of the conversion intermediates des-31,32-split proinsulin I and II, and des-64,65-split proinsulin I and II (reading from left to right); tCP, truncated (des-(27-31)-) C-peptide.

time for Hub to exert its dominant-negative effect on clathrin function.

Pulse-chase experiments and HPLC analysis of radioactive products

Cells were infected and sorted as above. They were then pulse-labelled (10 minutes [^{3}H]leucine). The fate of newly synthesized (radioactive) proinsulin was then followed during a subsequent chase. The first 150 minutes of chase were performed under basal conditions in order to keep secretion from secretory granules of the regulated pathway to a minimum. During this time period, any incorrectly sorted proinsulin would be secreted by the constitutive pathway. The medium was changed, and the cells were incubated for a further 60 minutes of chase in presence of a cocktail of secretagogues in order to stimulate (regulated) secretion of products stored in secretory granules. At the end of the second chase incubation, cells were extracted. Both the chase media and cell extracts were analyzed by reverse-phase HPLC in order to quantify radioactive proinsulin, insulin and C-peptide. A representative elution profile is shown in Fig. 2. Note that two nonallelic proinsulin genes are expressed in the rat. The HPLC protocol allows for separation of the two insulins and C-peptides but not of proinsulin 1 and 2. The minor peaks eluting before C-peptide have been shown to consist of the two C-peptide molecules lacking the five C-terminal residues (truncated, des-(27-31)-C-peptide) (see below).

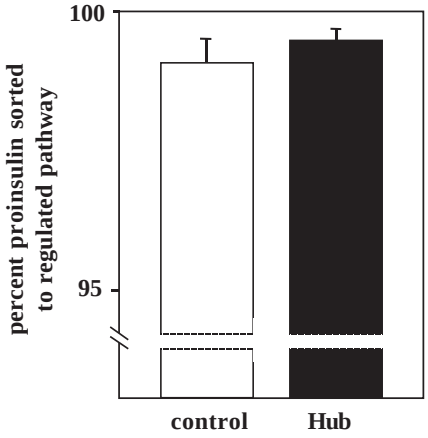

Fig. 3. Efficiency of sorting of proinsulin to the regulated secretory pathway is unaffected by Hub. Cells were infected with recombinant adenovirus expressing only EGFP (control) or EGFP and Hub (Hub). Those cells expressing the highest levels of EGFP (and thus Hub) were sorted by FACS and pulse-labelled for 10 minutes with [^{3}H]leucine before a 150 minute chase under basal conditions. Labelled proinsulin and insulin secreted during this time and that remaining within in the cells at the end of the incubation was quantified by HPLC (see Fig. 2). The percentage of labelled proinsulin directed to the regulated secretory pathway was calculated as 100−x, where x is the percentage of labelled proinsulin released without conversion during the 150 minute chase (and attributed to constitutive secretion) and is presented as mean±s.e.m., n=4. Note the interrupted scale. Sorting efficiency was >99% regardless of whether Hub was expressed in the cells or not.

Hub-expression does not perturb proinsulin sorting to the regulated secretory pathway, its conversion to insulin or the regulated secretion of proinsulin/insulin

The percentage of labelled proinsulin released by cells during the 150 minute basal chase (and considered to represent constitutive secretion) was less than 1% of total labelled proinsulin + insulin (basal and stimulated media + cell extracts). This indicates that both for cells expressing high levels of Hub and for controls, >99% of all newly synthesized proinsulin molecules were retained within the cell during this chase (Fig. 3). Clearly, the mere retention of proinsulin within cells does not constitute in itself any proof that these molecules were correctly sorted to and stored within secretory granules, although (along with the immunofluorescence staining pattern for proinsulin seen in Fig. 1) it is perfectly in keeping with this. It was thus necessary to investigate the conversion of proinsulin to insulin and C-peptide, an event known to arise within secretory granules and to quantify the proportion of proinsulin and/or insulin released from cells in response to secretagogues.

Proinsulin conversion was also found to be unaffected by Hub expression. Thus, the amount of fully processed insulin (expressed as a percentage of insulin + conversion intermediates + proinsulin) in basal medium, stimulated medium and cell extracts was not significantly different in Hub- vs control-infected cells (Fig. 4). The percentage of proinsulin/insulin released in response to secretagogues was similarly unaffected by Hub (Fig. 5). Thus, neither the very low levels of secretion under basal conditions, nor the marked stimulation of secretion by secretagogues (hallmarks of the

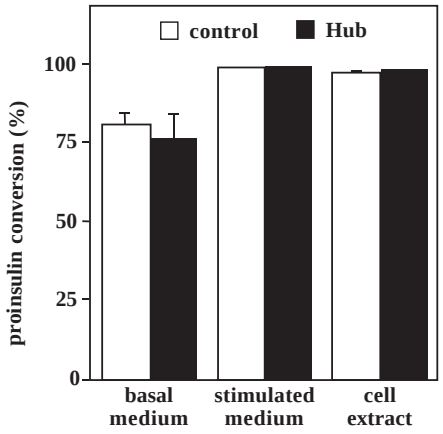

Fig. 4. Expression of Hub does not affect the conversion of proinsulin to insulin. Cells were infected with adenovirus, sorted and labelled as described in the legend to Fig. 3. After the 150 minute basal chase, cells were incubated for a further 60 minutes in the presence of secretagogues. The basal and stimulated chase media and extracts of cells at the end of the two chase periods were analysed by HPLC as described in Fig. 2. Proinsulin conversion (mean±s.e.m., *n*=4) is presented as labelled insulin/labelled proinsulin + intermediates + insulin x 100 (corrected for the difference in the number of leucines in each).

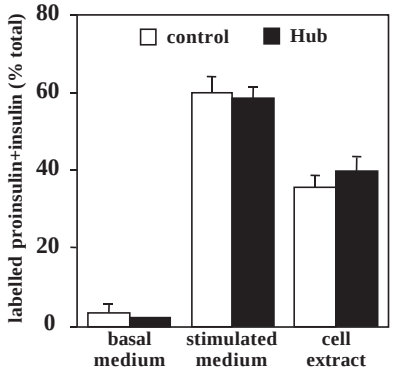

Fig. 5. Expression of Hub does not affect the release of proinsulin/insulin. Cells were infected with adenovirus, sorted and labelled as described in the legend to Fig. 3. Samples of medium, following a 150 minute incubation under basal conditions and a subsequent 60 minute period of stimulation, as well as cell extracts (after the stimulation) were analysed by HPLC (see Fig. 2). Labelled proinsulin and insulin (corrected for the difference in the number of leucines in each) is presented as a percentage of that found in all samples (basal + stimulated medium + extracts). Data are mean±s.e.m., *n*=4.

regulated secretory pathway and typical of primary β-cells) were affected by expression of the Hub fragment.

It is concluded that sorting of proinsulin to granules and its subsequent conversion to insulin is unimpaired in the absence of clathrin-coating activity and that exocytosis of granules is similarly normal.

Hub expression increases both the percentage of C-peptide released in the form of truncated des-(27-31)-C-peptide during 150 minutes of chase under basal conditions and more extensive C-peptide degradation thereafter

It has been shown previously that C-peptide, which is produced in equimolar amounts with insulin by the conversion of proinsulin, can be truncated. This truncation event results in loss of the end five (C-terminal) residues to give rise to des-(27-31)-C-peptide (Verchere et al., 1996). Such truncation arises within granules by an as yet unidentified protease. There is further and more extensive degradation of C-peptide, which occurs either in granules or subsequent to its vesicular transfer to lysosomes. Insulin, by contrast, is stable within granules. Thus, together, truncation and degradation of C-peptide lead to insulin:C-peptide ratios greater than unity.

The relative amounts of intact and truncated C-peptide and the ratio of insulin to total (intact + truncated) C-peptide was examined in the media. In the 150 minute basal medium, the percentage of C-peptide in the form of des-(27-31)-C-peptide was doubled (from 5 to 10%) in cells expressing Hub (Fig. 6). There was no such difference in the stimulated medium. By contrast, the ratio of insulin to total detectable C-peptide in the basal medium was not affected by Hub expression, whereas it was significantly elevated in the stimulated medium (Fig. 7A).

It is believed that truncation is the earliest proteolytic event to which C-peptide is subject in granules, followed by more extensive degradation to products no longer identified by the HPLC method used for analysis. Given the known stability of insulin within granules (Halban et al., 1987; Neerman-Arbez and Halban, 1993), if C-peptide is only truncated but not subject to more extensive proteolysis, the ratio of insulin to total detectable (intact + truncated) C-peptide will be unity. Further degradation will result in an increased ratio. The ratio of radiolabelled insulin:total C-peptide of 1.28 in the stimulated medium from cells expressing Hub (Fig. 7A) indicates that 21±2.3% of C-peptide had been degraded. We have shown that such C-peptide degradation occurs within cells and not in the medium following release from primary islet cells (Neerman-Arbez and Halban, 1993). Taken together, and given the sequential nature of the experimental protocol (150 minute basal secretion followed by 60 minutes of stimulation), the data presented in Figs 6 and 7A thus suggest that expression of Hub leads to both an increase in C-peptide truncation (as an early event picked up in the basal medium) and degradation (a later event impacting on insulin:total C-peptide ratio in stimulated medium).

It was predicted from the above data that the ratio of insulin to C-peptide in the steady-state should be elevated in cells expressing Hub. Unfortunately, there is no reliable analytical method available for direct measurement of rat C-peptide. We therefore resorted to an indirect method for measuring the insulin:C-peptide levels in β-cells. Cell extracts were analyzed by HPLC exactly as described for pulse-chase experiments. The absorbance of the HPLC column effluent was continuously monitored at 213 nm and the peak areas for insulin I and C-peptide I measured (note that steady-state levels of truncated C-peptide were too low to be routinely measurable by U.V. absorbance). The relative extinction coefficient of rat C-peptide vs insulin is not known. Given that we have shown only very limited C-peptide degradation in primary rat β-cells (Neerman-Arbez and Halban, 1993), the results (Fig. 7B) were normalized for an insulin:C-peptide ratio of 1:1 in control cells. The ratio was increased by 58% in cells expressing Hub,

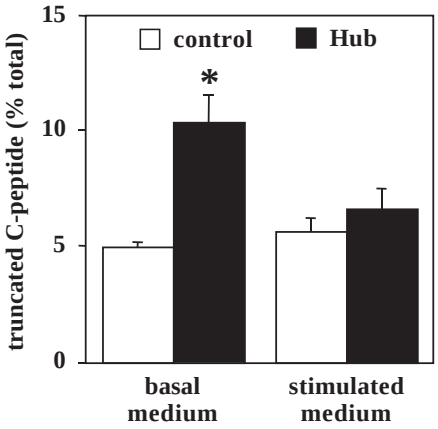

Fig. 6. Expression of Hub increases truncation of C-peptide. Cells were infected with adenovirus, sorted and labelled as described in the legend to Fig. 3. Radioactive truncated (des-(27-31)-) and intact C-peptide were quantified by HPLC (see Fig. 2). Truncated C-peptide is expressed as a percentage of total detectable labelled C-peptide (intact + truncated). Data are mean±s.e.m., n=4. *P<0.01 for Hub vs control for products released during the 150 minute basal chase.

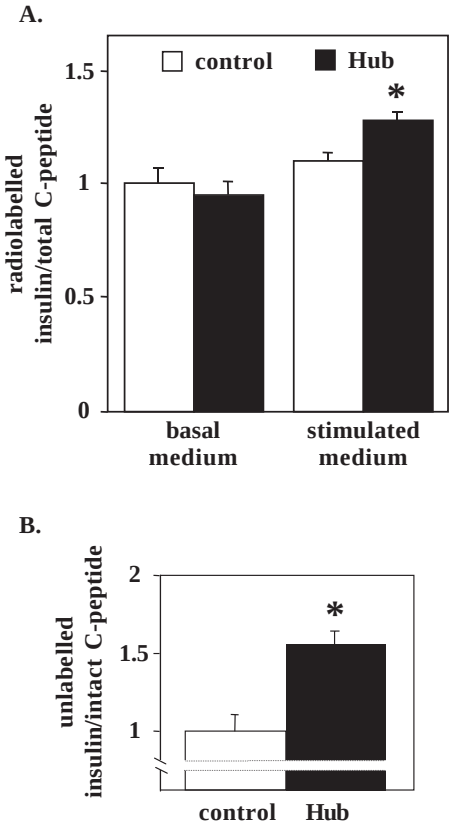

Fig. 7. Expression of Hub increases C-peptide degradation (relative to insulin) at later times of chase and in the steady state. Cells were infected with adenovirus and sorted as described in the legend to Fig. 3. (A) Pulse-chase. The sorted cells were labelled (10 minutes [^{3}H]leucine) and chased (150 minutes basal followed by 60 minutes stimulated). The two chase media were analyzed by HPLC. The data (mean±s.e.m., n=4) are for the ratio of labelled insulin to total (intact plus truncated) labelled C-peptide (corrected for the difference in the number of leucines in each). *P<0.02 for Hub vs control for products released during the 60 minute chase with secretagogues ('stimulated'). (B) Steady-state. The sorted cells were incubated for 150 minutes under basal conditions and then for a further 60 minutes with secretagogues. In order to estimate the ratio of (unlabelled) insulin:C-peptide, the 60 minute stimulated medium was analysed by HPLC and the peaks monitored by an online spectrophotometer at 213 nm. Peak areas for C-peptide I and insulin I were measured. The insulin:C-peptide ratio for cells expressing Hub was normalized to that for control cells (for which the measured value was 2.45±0.27). Data are presented as mean±s.e.m., n=5 from four independent experiments. *P<0.005.

confirming increased degradation of C-peptide relative to that of insulin in such cells.

Taken together, these data suggest that clathrin is normally implicated in maintaining C-peptide truncation and degradation at the low levels seen in primary β-cells (Neerman-Arbez and Halban, 1993; Verchere et al., 1996), presumably by virtue of its involvement in purging granules of unwanted proteases. In an attempt to document this, cathepsin B was examined by immunofluorescence. The enzyme has been shown by others to be found in granules as well as in lysosomes (Kuliawat et al., 1997) and co-localization of the enzyme with insulin was confirmed here (not shown). There was, however, no evident impact of the expression of Hub on either cathepsin B localization or levels (not shown). This negative result does not necessarily indicate that Hub was without effect on the trafficking of this or any other enzyme for the following reasons: (1) immunofluorescence only provides an indication of steady-state levels and not of fluxes; (2) the resolving power of light microscopy is not adequate for discrimination between lysosomes and granules. This is a confounding factor in the analysis given that insulin itself is known to reside in lysosomes in addition to granules (Orci et al., 1984c); (3) cathepsin B may not be a relevant marker as it has not been implicated in the truncation or degradation of C-peptide. More detailed analysis of the kinetics of passage of candidate enzymes through granules on their way to endosomes/lysosomes would thus be necessary to address this question.

Hub expression does not affect the amount of C-peptide released via the postgranular constitutive pathway

Secretion of C-peptide during the 150 minute basal chase period can be attributed to the combination of true basal exocytosis of large dense-core granules of the regulated pathway and so-called 'postgranular constitutive' or 'constitutive-like' secretion (Arvan and Castle, 1992; Arvan and Castle, 1998). This latter secretory pathway, first postulated for β-cells by Arvan and colleagues (Kuliawat and Arvan, 1992), involves the budding of vesicles from granules, taking with them soluble granular components including C-peptide, followed by constitutive exocytosis of such vesicles. Based on the assumption that insulin should be excluded from such vesicles, it is possible to estimate the amount of C-peptide released uniquely via the postgranular constitutive pathway during the 150 minute basal chase (Neerman-Arbez and Halban, 1993). The percentage of total labelled C-peptide released in this way was vanishingly small, and there was no significant effect of Hub (0.05±0.03 vs 0.19±0.12%/150 minutes, Hub vs control).

DISCUSSION

Expression of the dominant-negative clathrin mutant Hub peptide has been used with success in the past to unravel the role of clathrin in intracellular trafficking in the Class II major histocompatibility pathway (Altschuler et al., 1999; Liu et al., 1998; Trejo et al., 2000). These earlier studies are considered as proof of principle of this dominant-negative strategy as confirmed by immunofluorescence in the present study. Immunostaining for clathrin light chain in control cells revealed a predominant perinuclear, punctate compartment. Based on previous studies by electron microscopy (Orci, 1982; Orci et al., 1984a), this is taken to reflect the presence of clathrin on the TGN and immature secretory granules. Such staining was no longer evident in cells expressing the highest levels of EGFP and thus Hub. In order to ensure that the biochemical studies were limited to this particular cell population, cells were first sorted on the basis of their EGFP-fluorescence. Given that both recombinant adenovirus and EGFP itself could have untoward effects on cell function, it is important to note that control cells were also infected with adenovirus and sorted according to EGFP fluorescence. It was also necessary to define the timing of the experiments with considerable care. Clathrin is known to be implicated in a number of intracellular trafficking pathways and potentially in membrane recycling. Prolonged inhibition of clathrin function could thus be expected to be detrimental to cell survival over time in culture. However, Hub must be expressed at high enough levels for a sufficiently long period of time in order to ensure that membranes are no longer clathrin-coated. The timing of the experiments described here respected these two requirements.

The fact that immature granules carry only a discontinuous, rather than a continuous, coat of clathrin is in itself suggestive that they are in a class of their own and distinct from bona fide clathrin-coated vesicles (CCVs). One cannot conclude from this observation alone that clathrin is irrelevant to granule formation. It could, on purely theoretical grounds, be implicated in two steps: sorting of granule proteins in the TGN and budding/scission of granules per se (Arvan and Castle, 1998; Halban and Irminger, 1994). The present study clearly excludes such roles. The sorting of proinsulin to the regulated secretory pathway was remarkably efficient (as reported by us previously; Rhodes and Halban, 1987) in these primary rat β-cells, with >99% of all newly synthesized proinsulin being correctly sorted in both Hub-expressing and control cells. This does not in itself preclude a role for clathrin in the sorting of other granule constituents, however unlikely. The fact that the kinetics of proinsulin conversion were similarly unaffected, however, certainly indicates that the conversion enzymes (PC2, PC3 and carboxypeptidase E) were correctly packaged in granules and that the granular ATP-dependent proton pump needed for acidification and in turn for activity of the conversion endoproteases (Orci et al., 1994; Rhodes et al., 1987) was similarly present and active in the granule membrane. Clathrin is thus not implicated in these key events in granule maturation and function. Finally, the data show clearly that clathrin is not needed for regulated exocytosis of either immature (proinsulin) or mature (insulin) granules.

If clathrin is not needed for the sorting of granular constituents, the formation of granules or their function as the proinsulin conversion compartment and exocytotic vehicle, what purpose could it serve in the regulated secretory pathway? It has been suggested that CCVs bud from maturing granules (see Arvan and Castle, 1998, for review). Such formation of CCVs was first proposed on the basis of morphological studies some 20 years ago (Orci, 1982). Subsequently, it has been proposed that some C-peptide, as a soluble constituent of the granule, is captured in CCVs as they form from maturing granules (Arvan et al., 1991; Kuliawat and Arvan, 1992; Kuliawat and Arvan, 1994). This may be the case in less well differentiated transformed insulin-secretory cell lines, but in the primary β-cell <1% of C-peptide is secreted in this fashion (Neerman-Arbez and Halban, 1993). We confirm this in the present study. Given these very low values, it was not surprising that no significant inhibitory effect of clathrin inactivation was discernible at this level. Certainly it was not stimulated. By contrast, the hypothesis that CCVs may transport unwanted proteases away from granules to an endosomal intermediate (Turner and Arvan, 2000) is captivating, well documented (albeit indirectly given that the vesicles themselves have yet to be isolated) (Klumperman et al., 1998; Kuliawat et al., 1997) and of potential physiological significance. Such passage of an enzyme into and then out of granules has also been documented for the proprotein convertase furin (Dittie et al., 1997). A predicted consequence of the inhibition of this vesicular pathway would be the elevation of proteolytic activity in granules. C-peptide provides a useful marker substrate for such activity. In particular, we have shown that it can be selectively truncated to lose its five C-terminal residues (Verchere et al., 1996). Significantly, such truncation has been shown to arise in granules and not subsequent to shunting of C-peptide to another degradative compartment (Verchere et al., 1996). The observation of a doubling of C-peptide truncation in the basal medium of cells expressing Hub is quite in keeping with a role of clathrin in the removal of the (as yet to be identified) truncation protease(s) from granules. Such truncation is followed by more extensive degradation of C-peptide and this was also elevated in cells expressing Hub.

In conclusion, clathrin is not essential for sorting of proinsulin within the TGN, for the formation of β-cell secretory granules, for proinsulin conversion or for the regulated exocytosis of either proinsulin or insulin. Clathrin does appear, however, to be important for purging granules of proteases as they mature.

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